DETECTION AND MOLECULAR CHARACTERIZATION OF ROTAVIRUS STRAINS ISOLATED FROM CHILDREN ATTENDING SELECTED HEALTH FACILITIES IN KIAMBU DISTRICT, KENYA

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DECLARATION

This thesis is my original work and has not been presented for the award of a degree in any other University. All sources of information have been acknowledged by means of reference.

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DEDICATION

To Elsie, my daughter for the inspiration you gave me to venture into this work after a severe rotavirus infection at your tender age.

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ACRONYMS AND ABBREVIATIONS

BSA	Bovine Serum Albumin
cDNA	Complementary DNA
CTL	Cytotoxic T lymphocyte
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
ELISA	Enzyme Linked Immuno-sorbent Assay
EM	Electron Microscopy
Ig	Immunoglobulin
KCl	Potassium chloride
KNBS	Kenya National Bureau of Statistics
NSP	Non-structural protein
ORT	Oral Rehydration Therapy
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PBST	Phosphate buffered-saline containing Tween 20
RNA	Ribonucleic acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RPM	Revolutions per minute
T_H	Helper T lymphocyte
VP	Viral protein
WHO	World Health Organization
M	Molar
ml	Millilitre
mM	Millimolar
µl	Microlitre

ABSTRACT

Despite numerous health intervention measures available, severe dehydrating rotavirus diarrhea remains a major contributor towards childhood mortality particularly in developing countries. Global rotavirus surveillance is vital towards the development of safe, effective and efficacious vaccines to control the associated high infection rates. In Kenya, however, there is little corroborated data on rotavirus epidemiology, burden of disease and strains in circulation. The objective of this study was to determine the prevalence and molecular characteristics of rotavirus strains responsible for severe gastroenteritis in children in Kiambu District, Kenya. A total of 232 fecal samples were collected between August 2008 and May 2011from children below 5 years old with diarrhea hospitalized at Kiambu District Hospital andKaruri Health Centre. The specimens were screened for group A rotavirus usingEnzyme Linked Immuno-sorbent Assay(ELISA). RNA from ELISA-positive specimens was separated bypolyacrylamide gel electrophoresis(PAGE) to determine rotaviruselectropherotypes. Reverse Transcription Polymerase Chain Reaction(RT-PCR) was used to determine rotavirus G and P genotypes. The ELISA screen gave 36.6% positive results for group A rotavirus among the diarrheal cases. Rotavirus was detected most frequently in infants and young children aged below 2 years with a peak at 6 to 11 months (χ^2 = 12.162; df = 4; P = 0.016). The virus was found year-round with slight peaks and valleys insome months(χ2 = 96; df =90; P value = 0.313). Of the 85 ELISA-positive samples, 58 (68.1%) gave visible RNA profiles whereas 28 (32.9%) gave invisible profile. Of the visible RNA profiles, 92.9%, 5.3% and 1.8% displayed long, short and more than 11 RNA segmentselectropherotypes respectively (χ^2 = 344.621; df = 1; P = 0.001). Five different G genotypes were determined in 55 of 85 of the specimens analysed (χ^2 = 447.48; df = 1; P = 0.001). G1 was predominated among the strains at 44.7%. Other usual global genotypes; G2, G4 and G9 were detected at10.6%, 4.7% and 1.2% respectively. G8, an African-specific strain was isolated at 8.2%. Three different P genotypes were determined in 55.3% of the specimens analysed (χ^2 = 376.379; df = 1; P = 0.001).P [8] and P[4] predominated at 28.2% and 25.9% respectively. P[6], an African-specific strain was isolated in one sample. Data generated from this study will add crucial information on the burden of the rotavirus disease and genotype distribution in the country. Such information will not only aid in seeking advocacy for rotavirus vaccine introduction in the country's national immunization programme, but will also help in the evaluation of the efficacy of these vaccines in relation to the rotavirus genotypes in circulation. The heterogeneity and ever-changing epidemiology of rotavirus observed in this and other related studies underscores the need for continued surveillance of rotavirus strains throughout Kenya to ensure that vaccination programmes being advocated for provide optimal protection.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Diarrhoeal diseases account for approximately 17% of the 10.4 million deaths among children aged below 5 years globally (Parashar *et al*., 2006). Among children less than 5 years of age, rotavirus infection is the leading cause of moderate to severe acute diarrhoeal disease, accounting for an estimated 527,000 deaths annually (WHO, 2009). About 85% of these deaths occur in the poorest developing countries in Africa and Asia, defined as "low-income" by the World Bank due to lack of timely and appropriate treatment for dehydration (Patel *et al*., 2009). The current estimated annual death toll due to rotavirus gastroenteritis in Africa alone is between 150,000-200,000, with more than 7,500 of these deaths occurring in Kenya (Nyangao *et al*., 2010; Mulholland *et al.*, 2008). Rotavirus is also the most common cause of hospitalizations for diarrhoea, reflecting a significant cost in health resources. Various studies have found that in developing countries, rotavirus accounts for approximately 8% of all diarrheal episodes, 28% of clinic visits for diarrhoea, and 34% of hospitalizations of young children for diarrhoea (Parashar *et al.*, 2006; Leung *et al.*, 2005).

The burden of disease due to rotavirus gastroenteritis can be reduced by improving sanitation and educating parents on rehydration therapy to prevent child mortality and morbidity from dehydration and developing vaccines to prevent the disease (O'Ryan *et al*., 2009). Improving sanitation is complicated by poor infrastructure and funding in many developing countries where rotaviraldiarrheoa is particularly devastating. Notably, standard sanitary measures that kill most

bacteria and parasites are ineffective in controlling rotavirus, and because low numbers (10-100 particles) of viruses can cause infection, transmission is common even with good hygiene practices (Parashar *et al*., 2003). This is demonstrated by the fact that rotavirus incidence is similar in countries with both high and low sanitation standards (CDC, 2008; Kane *et al.*, 2004; Kang *et al.,* 2005; Kiulia *et al.,* 2008).Intravenous treatments which are effective against severe dehydration are largely unavailable to the developing world's children under age five. While the alternative treatment of oral rehydration therapy (ORT) is more available, there are still significant setbacks in its distribution or instructions for its production in the developing world (Madhi *et al*., 2010).

Preventing rotavirus gastroenteritis through vaccination would be a much more high-impact and cost-effective public health intervention tool to greatly reduce the number of deaths due to diarrheal diseases, greatly reduce the burden on the health system and to achieve Millennium Development Goal 4 (Cunliffe and Nakagomi, 2005; Tessa *et al.,* 2010; Rodrigo *et al*., 2010).However, the World Health Organization (WHO) requires that the efficacy of a rotavirus vaccine be demonstrated specifically in low-income countriesof Africa and Asia, before it recommends its inclusion in the global program for childhood immunization. In addition, the GAVI Alliance (formerly known as the Global Alliance for Vaccines and Immunization) will assist the developing countries in financing introduction of a rotavirus vaccine only if its efficacy is demonstrated in the region (Patel *et al.,* 2009).

In 2006, two vaccine candidates were developed: the Merck vaccine RotaTeq composed of five bovine–human reassortant strains including G types G1–G4 and P type P1A[8] and the GlaxoSmithkline vaccine, Rotarix including one human attenuated P1A[8]G1 strain (O'Ryan *et*

al., 2009). The efficacy of these vaccines is dependent on the elicitation of serotype-specific, heterotypic, or a combination of serotype-specific and heterotypic immunity to the globally predominant rotavirus serotypes (Patel *et al*., 2009; Ruiz-Palacios *et al.,* 2006). However, there is increasing molecular epidemiology data indicating a regional diversity of rotavirus 'serotypes' in circulation. The number of VP4/VP7 antigenic combinations possible is large considering that at least 12 G and 11 P types have been detected among human rotaviruses (Santos and Hoshino, 2005; Nyangao *et al*., 2010). Besides, there is emergence of serotypes across the African continent with different VP4/VP7 antigenicity as a consequence of animal-human virus reassortment which may not be cross-protective with the current vaccine types (Gentsch *et al.*, 2005; Page *et al.,* 2010). In view of this, strain characterization, along with burden data are critical to support an informed and evidence-based decision about necessity of introducing rotavirus vaccines in a country and suitability of a particular vaccine with regard to the genotypes circulating in the country (Hoshino *et al*., 2004; O'Ryan *et al*., 2009; Rodrigo *et al*., 2010).

1.2 Problem Statement and Justification

In Kenya, rotavirus surveillance work has been done only in few parts of the country. As a result, there is currently little corroborated data on rotavirus epidemiology and burden of disease as well as the strains circulating in the country. Moreover, no studies on prevalence and molecular epidemiology of rotavirus have been carried out in Kiambu District of Kenya (Kiulia *et al.,* 2006; Nyangao *et al.,* 2010; Mulholland *et al.*, 2008). This study, therefore, aimed at determining the prevalence and molecular characteristics of the rotavirus strains isolated from children attendingselected health facilities in Kiambu District of Kenya. It was envisaged that the data

generated from this project wouldadd crucial information on the importance of rotavirus infections in the overall burden of diarrheoal diseases in children below the age of 5 years, and the genotypic distribution of rotavirus in this region of Kenya. Such information would aid in seeking advocacy for vaccine introduction in the country's national immunization program and also to help evaluate the efficacy of these vaccines in relation to the genotypes in circulation (Nyangao *et al*., 2010).

1.3 Hypotheses of the Study

i. Rotavirus infections exhibit high prevalence rate and age and seasonal patterns among children attending selected health facilities inKiambu District, Kenya.
ii. There's diversity of rotavirus electropherotypes circulating among children attending selected health facilities in Kiambu District, Kenya.
iii. There's genotypic diversity among rotavirus strains isolated fromchildren attending selected health facilities inKiambu District, Kenya**.**

1.4 Objectives

1.4.1 General Objective

To determine the prevalence and molecular characteristics of the rotavirus strains detected in children admitted to health facilities in Kiambu District, Kenya.

1.4.2 Specific Objectives

i. To determine the prevalence, seasonality and age distribution of rotavirus infections in children attending selected health facilities in Kenya using ELISA.

ii. To determine the electropherotypic patterns of rotavirus strains detected in children attendingselected health facilities in Kiambu District, Kenya using PAGE.

iii. To determine the genotypic diversity of rotavirus strains detected in children attendingselected health facilities in Kiambu District, Kenya using RT-PCR.

CHAPTER TWO

LITERATURE REVIEW

2.1 Discovery of Rotavirus

Rotavirus was discovered in 1972 by an Australian research group led by Dr. Ruth Bishop. The virus was recognized by direct electron microscopy visualization in the duodenal biopsies of a child with acute diarrhoea, and named duovirus(Bishop *et al.,* 1973). The virus was subsequently observed in large numbers in faeces as demonstrated by direct thin layer electron microscopy and significant antibody titre was shown between acute and convalescent sera from the children by immune electron microscopy (Bishop *et al.,* 1974). The virus was renamed rotavirus because of its characteristic wheel-shaped (rota is latin for wheel) morphology when viewed under an electron microscope (Prasad and Chiu, 1994).

2.2 Geographical Distribution and Epidemiology of Rotavirus

Rotavirus is distributed evenly across the globe. Regardless of hygiene practices or access to clean water, nearly every child in the world will be infected with rotavirus before age five (Parashar *et al*., 2003). However, the consequences of infection are markedly severe depending on where the child lives and the majority of deaths due to rotavirus diarrhoea occur in the developing countries of the Indian subcontinent and sub-Saharan Africa due to limited access to medical intervention (Parashar *et al.*, 2006;Cunliffe *et al.*, 2005).

Humans of all ages are susceptible to rotavirus infection, but children aged 6 months to 2 years, premature infants, and the elderly and immuno-compromised individuals are particularly prone to more severe symptoms. Children become most susceptible after 6 months of age when the

protection afforded by maternal antibodies wanes (Patel *et al*., 2009). The median age of children hospitalized with rotavirus diarrhoea in many African and Asian countries is 6-9 months, and up to 80% are less than 1 year old (Cunliffe *et al.,* 1998). In contrast, the median age in developed countries is 13-16 months and the highest proportion of cases occurs in the second year of life (Nakagomi *et al.,* 2005). By 15 months of age many have developed some protection after primary infection (O'Ryan *et al*., 2009). Nevertheless, in both developing and developed countries, rotavirus is the major cause of severe gastroenteritis and is associated with approximately 40% of hospitalizations worldwide (CDC, 2008).

High transmission rates of rotavirus have been associated with the dual condition of extremely high virus concentration in faeces of symptomatic and asymptomatic individuals (more than 10^9 virus particles/g) and the low inoculums required for infection (10–100 virus particles). Widespread viral contamination of different water bodies (with the possible exception of seawater) and prolonged persistence of infective virus in ground and surface water may be contributing to the high prevalence rates of rotavirus infection worldwide (Grassi *et al.,* 2009;Espinosa *et al.,* 2008). In temperate countries, rotavirus infections peak in the winter and early spring, with fewer cases at other times. In tropical countries, rotavirus infections occur throughout the year, although more cases are observed in the cooler and drier months (Nakagomi *et al.,* 2005).

Molecular epidemiological studies of rotavirus have identified 5 common serotypes, including G1, G2, G3, G4, and G9, which tend to predominate globally (Desselberger *et al.,* 2001;Santos and Hoshino, 2005). G1 is the most prevalent strain worldwide whereas G9 is the fastest emerging worldwide (Page *et al*., 2010;Nyangao *et al.,* 2010;Kirkwood *et al*., 2003). However,

in developing countries, additional serotypes may circulate and even predominate in some setting (eg, G5, G8, G10, and G12). Of the 27 VP4 genotypes identified, genotypes P1A[8], P1B[4], P2A[6], P3[9], P4[10], P5A[3], P8[11], P12[19], P[25], and P[28]are detected most frequently in children (Hoshino *et al.*, 2000;Santos and Hoshino, 2005). Analogously to VP7 epidemiology, supplementary P genotypes, including P[6], P[9], and P[10], may also predominate or circulate at lower levels in developing countries (Santos and Hoshino, 2005).

2.3 Virology of Rotavirus

2.3.1 Structure and Taxonomy

Rotavirus is a non-enveloped virus of the family *Reoviridae* (Anderson and Weber, 2004). It has a wheel-like appearance on electron microscopy (Prasad and Chiu, 1994). The virus has a triple-layered icosahedral capsid 76.5 nm in diameter and has a buoyant density of 1.36 g/ml in CsCl(Maldonado and Yolken, 1990;Pesavento *et al.,* 2006).

2.3.2 Genome

Rotavirus genome is made up of 11 segments of double stranded RNA (dsRNA) held in the inner core of the three-layered virus (Varani and Allain, 2002). The genome consists of 18,555 nucleotides in total. Each segment is a gene, numbered 1 to 11 by decreasing size. The segmented genome can be separated by polyacrylamide gel electrophoresis (PAGE) to reveal an RNA migration pattern or electropherotype. The RNA pattern is both constant and characteristic for a particular strain, and has been widely used in epidemiological studies, for monitoring the transmission and spread of rotavirus (Steele *et al*., 1993). Each of the 11 segments of

dsRNAcodes for one of six structural and six nonstructural proteins, with only segment 11 being bicistronic (encoding two proteins) (Anderson and Weber, 2004).

2.3.3 Proteins

The six viral proteins (VP1, 2, 3, 4, 6 and 7) form the virus particle (virion). VP1 is the RNA-Dependent, RNA Polymerase for rotavirus (Varani and Allain, 2002;Rodrigo Vasquez-del Carpio *et al*., 2006). VP2 is a replication intermediate and binds the RNA genome while VP3 acts as the mRNA capping enzyme called guanylyltransferase(Fresco and Buratowski, 1994).VP4 determines the rotavirus P serotype, as well as host specificity, virulence and protective immunity (Maunula and Von Bonsdorff, 2002). VP7 is a glycoprotein that determines the G serotype. VP6 determines the A-G groupings, and I, II sub-groupings of rotavirus (Laird *et al*., 2003).

The six non-structural proteins (NSP1, 2, 3, 4, 5 and 6) are only produced in cells infected by rotavirus (Graff *et al*., 2002;Anderson and Weber, 2004). NSP1 binds Interferon Regulatory Factor 3 and may inhibit interferon response during rotavirus infection (Graff *et al*., 2002). In conjunction with NSP5, NSP2 is involved in the synthesis and packaging of viral RNA, creation of viroplasms and is required for genome replication. NSP3 binds viral mRNA at the 3' end, promotes viral protein synthesis and is responsible for the shutdown of host cell protein synthesis. NSP4 is a viral enterotoxin and induces diarrhea during infection (Dong *et al*., 1997). NSP6 is an RNA binding protein encoded by gene 11 from an out of phase open reading frame(Rainsford and McCrae, 2007).

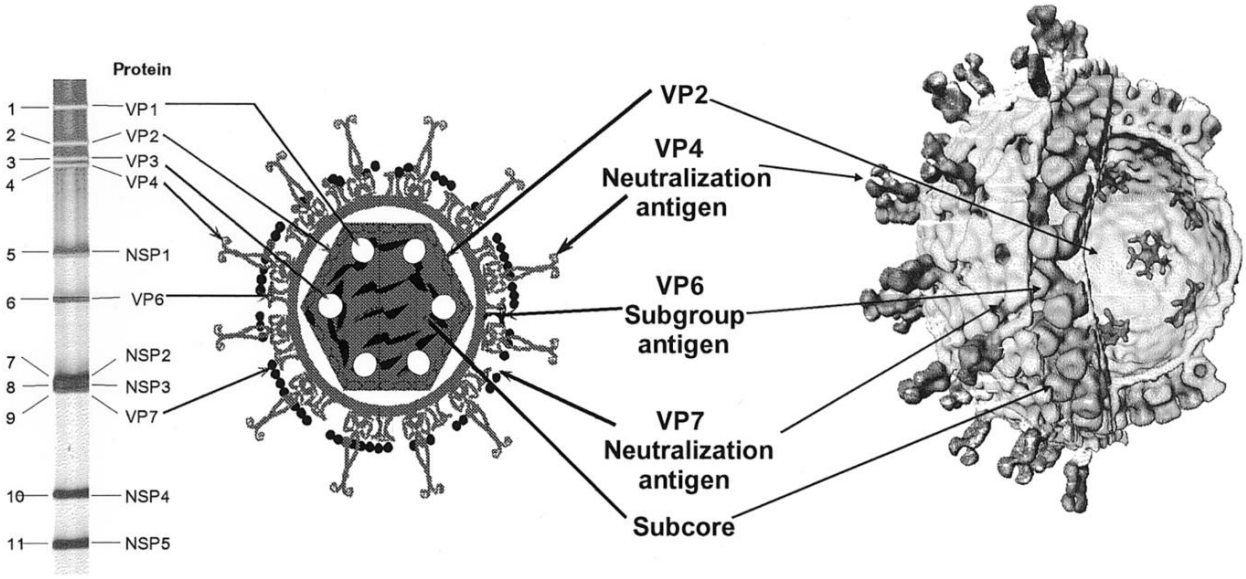

Figure 1. Rotavirus structure showing protein coding assignments of 11 genome RNA segments separated on polyacrylamide gel *(left)*. Schematic diagram *(middle)* and cryoelectron microscopic reproduction of a virion*(right)* show the location of major structural proteins (VP). Outer capsid proteins VP4 and VP7 are neutralization antigens, which induce neutralizing antibody; protein that makes up intermediate protein shell, VP6, is the subgroup antigen. NSP, nonstructural protein (Gentsch*et al.,* 2005).

2.3.4 Replication

Rotavirus entry into enterocytes is accompanied by the loss of the VP4 and VP7outer layer, thereby converting the triple-layeredparticles (TLPs) to double-layered particles(DLPs). The RNA-dependentRNA polymerase (RdRp) VP1 of the DLP functions as a transcriptase tosynthesize the 11 viral plus-strand RNAs (Lawton *et al.*, 1997). The plus-strandRNAs are extruded from DLPs through channels at the verticesthat extend through both the VP2 and VP6 protein layers. Theplus-strand RNAs contain 5' caps but lack 3' poly(A) tails andare translated to give rise to six structural proteins and six nonstructural proteins. The plus-strand RNAsalso function as templates for the synthesis of the dsRNA genomesegments. RNA replication occurs concurrently with the packagingof the genome segments into newly formed cores and is

coordinatedsuch that the 11 segments are produced at equimolar levels (Patton and Gallegos, 1990;Patton, 1990).

Rotavirus infection leads to the formation of perinuclear, non-membrane-boundcytoplasmic inclusions (viroplasms). NSP2and NSP5 have critical roles in viroplasm formation (Lawton *et al*., 1997). Viroplasms are the putative sites of RNA replication(minus-strand synthesis) and core and DLP assembly. The DLPs migrate to the endoplasmic reticulum where they obtain their third, outer layer (formed by VP7 and VP4). The progeny viruses are released from the cell by lysis(Jayaram *et al*., 2004).

2.3.5 Classification

Within the Rotavirus genus, there are seven groups (A to G) based on the VP6 protein, each of which represents a separate species (Maldonado and Yolken, 1990). Only group A, B and C rotaviruses are established as human pathogens. Group A rotavirus has much greater medical importance and, unless otherwise mentioned, rotavirus usually means group A rotavirus (Anderson and Weber, 2004). Group B rotaviruses are common animal pathogens infecting pigs, cows, sheep and rats, but have also been found infecting both adults and children, causing both outbreaks and sporadic infections, primarily in China, India and Bangladesh (Kang *et al.*, 2005). Group C rotaviruses, commonly found in animals including pigs and dogs, can cause outbreaks in the human population, especially older children, and up to a third of adult humans have serological evidence of infection with group C rotavirus (Hoshinoand Kapikian, 2000).

Within group A, rotaviruses are classified into subgroups I, II, I+II, nonI, and nonII based on the subgroup antigens that are carried on the VP6 protein (Greenberg *et al*., 1983). Further discrimination within group A rotaviruses into different strains, called serotypes is based on two

outer capsid proteins VP7 and VP4 (Martinez-Laso *et al*., 2009). VP7 (G protein for 'glycoprotein' forming the matrix of the capsid) defines G serotypes. VP4 (P protein for 'protease-sensitive' due to its trypsin mediated cleavage required for virus adsorption into cells) determines the P serotypes (Inoue *et al*., 2003;Laird *et al*., 2003). For G types, serotypes (determined by neutralization assay) and genotypes (determined by RT-PCR) are largely identical, thereby allowing the use of the same numbering system, but for P types, more genotypes than serotypes have been identified, owing to lack of monospecific P antisera. As a result, P types are identified as serotypes by Arabic numbers and as genotypes by Arabic numbers in square brackets. Thus, the serotype of prototype human rotavirus strain Wa is described as G1P1A[8]. To date, 20 distinct G types (G1–G15) and at least 27 P types (P[1]–P[26]) have been found in humans and animals (Matthijnssens *et al*., 2008;Santos and Hoshino, 2005). However, the G and P type combinations detected in human rotaviruses are mostly limited to G1P1A[8], G2P[4], G3P[8], G4P[8] and G9P[8] (Solberg *et al*., 2009).

2.4 Pathogenesis of Rotavirus

The primary mode of person-to-person transmission of rotavirus is faecal-oral, although some studies have reported low titers of virus in respiratory tract secretions and other body fluids, indicating the possibilities for air-borne and water-borne transmissions of rotavirus(Dennehy, 2000). After ingestion, the rotavirus particles exclusively infect the mature differentiated enterocytes in the mid and upper part of the villi of the small intestine, leading to structural changes in the intestinal epithelium(Lundgren and Svensson, 2001). Unlike the parvovirus, rotavirus can infect neither the immature villous crypt cells nor the colonic enterocytes. Rotavirus attaches to its cellular receptors (sialoglyco-protein and integrins) via the VP4 protein.

The virus is thought to invade target cells in two possible ways; by direct entry or fusion with the enterocytes, and through Ca^{2+}-dependent endocytosis (Pérez *etal*., 1998; Jayaram*et al.*, 2004).

Three mechanisms have been described by which rotavirus might cause diarrhoea. First, within 12-24 hours post-infection, enterocytes are intact but the levels of the brush-border dissacharidases (sucrase, maltase, lactase) are greatly reduced. As a result, dissacharides in the diet cannot be hydrolysed to monosaccharides and thus cannot be absorbed, leading to osmotic diarrhoea (Anderson and Weber, 2004). Second, NSP4 has an effect in opening calcium channels in the enterocytes. This causes an efflux of sodium and water, producing secretory diarrhoea(Dong *et al.*, 1997). Finally, the raised intra-enterocyte calcium concentration causes enterocytes to die by oncosis. The rate of death of the mature villous tip enterocytes exceeds the rate of growth of immature enterocytes that are regenerated from the stem cells in the crypt, causing villous blunting and thus malabsorption (Leung *et al.*, 2005). Infection resolves both as the virus runs out of susceptible mature enterocytes and an immune response is generated(Lundgren and Svensson, 2001).

2.5 Immune Response to Rotavirus

Primary rotavirus infections induce production of rotavirus-specific memory B and T cells (Velazquez*et al.*, 2000). However, in humans, high titers of IgG do not seem to be as protective as IgA against moderate to severe illness, so serum IgA is seen as the primary indicator of protective immunity to rotavirus. One reason these antibody responses do not confer full protection is that they are serotype specific. Given the diversity of the various rotavirus serotypes, this prevents these antibodies from mediating full protection against infection by a different serotype. However, each additional infection expands the population of B cells

producing cross-reactive antibodies that can recognize multiple serotypes and explains why repeat infections are less severe. Any vaccine effort would need to generate these cross-reactive antibodies to generate effective protection(Rodrigo *et al.*, 2010).

$CD4^+$ helper T (T_H) cells also play a vital role in the successful clearance of a rotaviral infection (VanCott*et al.*, 2001). Thus the correlates of immunity to rotavirus include both the presence of high amounts of cross reactive secretory IgA, and serotype specific serum IgA and IgG, which requires a rotavirus-specific T_H cell response, as well as a rotavirus-specific CTL response (Anderson and Weber, 2004).Protection of neonates against rotavirus infection appears to be conferred by both transplacentally acquired maternal antibodies and by antibodies and other factors in breast milk. Interestingly, rotavirus infection in neonates often results in asymptomatic infection unless novel serotypes emerge, and rotavirus can circulate silently in neonatal units(Patel *et al*., 2009).

2.6 Clinical Features of Rotavirus Infection

The outcome of rotavirus infection varies from asymptomatic through mild short-lived watery diarrhoea, to an overwhelming gastroenteritis with dehydration leading to death. The onset of symptoms is abrupt after a short incubation period of 1-3 days. The disease is characterized by fever, frequent abdominal pain and vomiting for 2-3 days, followed by pale watery or loose non-bloody diarrhea for 3-8 days. Diarrhoea can be profuse, with patients commonly having 10-20 bowel movements each day. Such severe diarrhea without fluid and electrolyte replacement may result in death.Temporary lactose intolerance may also occur. Respiratory signs are often found during rotavirus gastroenteritis but its aetiological association with rotavirus infection is not clear. It has been recently shown that rotavirus gastroenteritis may lead to extra-intestinal

manifestations such as viraemia. Patients continue to excrete virus for extended periods of time and may thus be a reservoir for infecting others (Maldonado and Yolken, 1990).

It is not possible to distinguish rotavirus gastroenteritis from other viral causes of non-inflammatory diarrhoea solely on clinical grounds(Lundgren and Svensson, 2001). However, rotavirus diarrhoea tends to be more severe than that due to other enteropathogens. Co-infection with another pathogen does not increase the severity of disease due to rotavirus infection(Leung *et al.,* 2005).

2.7 Diagnosis of Rotavirus

Diagnosis of rotavirus can be done by identifying the virus in the patient's stool using techniques such as antigen detection assays, electron microscopy (EM), polyacrylamide gel electrophoresis (PAGE), reverse transcription-polymerase chain reaction (RT-PCR) and virus isolation(Cunliffe*et al.*, 2002). Antigen detection tests are the most widely used in diagnostic laboratories and include enzyme-linked immunosorbent assay (ELISA), latex particle agglutination assay (LA) and immunochromatography (Smith *et al.,* 1993). Though the sensitivity and specificity of these tests are generally high, they are only designed to detect group A rotavirus. Furthermore, ELISA is not a reasonable method after day 10 post-infection when antibody levels in the stool drop(Greenberg*et al.,* 1983). Other groups of rotavirus can be isolated in cell cultures but viral culture is limited to research purposes. Antibody detection may also be employed for rotavirus diagnosis but is not commonly used. EM is relatively quick and can be used to identify non-group A rotaviruses (Bishop *et al.,* 1974). However, access to electron microscopes is not usually available in developing nations. PAGE is convenient for the detection of rotavirus RNA extracted directly from the stool specimens. The assay also allows

detection of non-group Arotaviruses. The technique is relatively cheap and simple with good specificity and sensitivity. In addition, this assay provides epidemiological information based on the electrophoretic migration pattern of the 11 segments of the dsRNA(Matthijnssens*et al.*, 2008). RT-PCR is generally considered the standard tool in virus detection for research purposes (Gouvea*et al.*, 1990). The technique provides information on the G and P genotypes of the circulating rotavirus strains and the duration of viral shedding in the stool(Fischer and Gentsch, 2004).

2.8 Management, Prevention and Control of Rotavirus Infection

There is no cure for rotavirus. Therefore, the mainstay of management involves replacement of lost fluid by oral rehydration with fluids of specified electrolyte and glucose composition (Anderson and Weber, 2004). Intravenous rehydration therapy is indicated for patients with severe dehydration, shock or reduced levels of consciousness. Human or bovine colostrum and hyperimmune human serum immunoglobulin may be used to manage chronic rotavirus infection in immunocompromised children. Administration of probiotics such as *Lactobacillus casei* GG may also be beneficial. Anti-diarrheal medicines are not recommended because they may prolong the infection (Leung *et al.,* 2005).

Following the magnitude of disease associated with rotavirus infections and because public health interventions to improve sanitation are unlikely to decrease the incidence and burden of this disease, vaccines are being developed as the first line strategy for prevention (O'Ryan *et al*., 2009; Rodrigo *et al*., 2010).Several clinical studies in children including well designed cohort studies have conclusively demonstrated that a natural rotavirus infection protects against reinfection but protection is incomplete (Velasquez *et al.,* 1996; Fischer *et al.,* 2002). Children

(and probably adults) can be reinfected many times throughout the years but the great majority will suffer at most one moderate to severe clinical episode during the first encounter with the virus. This clinical observation was the basis for the concept of 'infection induced protective immunity' leading to the concept that 'vaccine induced protective immunity' could be obtained (Rodrigo *et al*., 2010).

Proof of the concept of 'infection induced protective immunity' was provided in 1998 with Rotashield, an oral formulation of a simian-human quadrivalentreassortant rotavirus vaccine manufactured by Wyeth Laboratories(Hochwald and Kivela, 1999). Protection was demonstrated in children at 2, 4 and 6 months but the vaccine was withdrawn from the market due to a significant association with intestinal intussusception. The mechanism of this rare association remains unknown although the higher frequency of adverse events associated with the rhesus rotavirus component and the low frequency of the event suggest an unusual, probably individual, susceptibility to this component in affected children(Patel *et al.*, 2009). Irrespective of the reason, the new vaccine candidates had to demonstrate lack of association with intussusceptions(Vesikari*et al.*, 2006). Two vaccine types, RotaTeq® and Rotarix®, have been prequalified by WHO and are being evaluated worldwide (Armah*et al.*, 2010; Zaman*et al.*, 2010).

2.9 Review of Rotavirus Research in Kenya

Early research on the etiology of diarrhoea in infants and young children in Kenya determined the importance of rotavirus infections in the overall burden of diarrheal diseases in children

below the age 5 years, established the general age distribution of children infected with rotavirus, and determined the seasonality of rotavirus infections (Leeuwenburg *et al.*, 1978; Mutanda *et al.*, 1984). A review of rotavirus studies conducted in Kenya between 1975 and 2005 indicated that the overall prevalence of rotavirus ranged from 11% to 56.2% in children below 5 years of age and 6% in neonates (Kiulia *et al*., 2008). A study conducted by Urasawa and colleagues in Kilifi and Mombasa between 1982 and 1983 employing ELISA and serotype-specific monoclonal antibodies demonstrated the diversity of rotavirus strains in Kenya (Urasawa *et al*., 1987). Serotype G1 was identified as the predominant strain, and mixed infections were also detected. In a similar study conducted in Nairobi, Nanyuki, and Narok between 1989 and 1991, serotype G1 strains was observed in the majority of specimens analyzed (Kiulia *et al*., 2008).

The first large-scale studies in Kenya were conducted in Kitui and Nanyuki between 1991 and 1993, and in Nairobi between 1991 and 1994 (Gatheru *et al.*, 1993; Nakata *et al.*, 1999). During the studies, the overall frequency of detection of rotavirus antigen in the specimens analyzed was 22.2%, although the prevalence differed between the areas of study. Serotype G4 was the most prevalent strain, followed by G1 and G2. G8 strains were identified for the first time in Africa, whereas G3 were rarely isolated. Further studies conducted in Nairobi between 1999 and 2000 revealed that G3 were the predominant strains, with G4, G8, and G9 circulating at lower levels. An additional study in Maua between 2004 and 2005 indicated G9 strains as the most prevalent strain, followed by G8 and G1 strains (Kiulia *et al.*, 2006).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Location

The study was conducted in Kiambu District in the Central Province of Kenya. The district is adjacent to the northern border of Nairobi and has a population of 1,623,282 according to the 2009 KenyaPopulation and Housing Census(KNBS, 2010). The stool samples were collected from Kiambu District Hospital and Karuri Health Centre both of which are run by the government of Kenya. Kiambu District Hospital has 316-bed general wards and 67 cots and generally serves populations from Kiambu district and its environs that include Nairobi. Karuri Health Centre has an 18-bed general ward and generally serves residents of Kiambu district.

3.2 Study Population

Study subjects were infants and young children below 5 years of age with severe diarrhea hospitalized at either Kiambu District Hospital or Karuri Health Centre.

3.3 Sampling

3.3.1 Sampling Method

Convenience sampling technique was used where all diarrheic children aged less than five years who met all the inclusion and none of the exclusion criteria and were hospitalized at either Kiambu District Hospital or Karuri Health Centre were selected.

3.3.2 Sample Size Estimation

The standard statistical approach to determination of sample size for a cross-sectional study such as this one required specification of an estimate of the proportion (prevalence); the desired level of confidence for the proportion estimate; and a tolerance error margin so that the necessary sample size was then calculable for a given precision level using the following formula:

$$n = \frac{t^2 \times p(1-p)}{m^2}$$

Where:

n= required sample size

t = confidence level at 95% (standard value of 1.96)

p = estimated global prevalence of rotavirus gastroenteritis of 40% (WHO, 2009)

m = margin of error at 7% (critical value of 0.07)

Thus, it was estimated that at least 188 samples would be necessary to achieve the required sufficient precision for the study. A total of 232 stool samples were available for this study.

3.3.3 Inclusion Criteria

Only children under 5 years of age who presented with acute diarrhea for not more than 7 days and having experienced an episode of 3 looser than normal or watery stools in a 24-hour period with or without episodes of vomiting were enrolled in this study (WHO, 2009).

3.3.4 Exclusion Criteria

Children more than 5 years of age and with diarrhea lasting for more than 7 seven days and having bloody diarrhea were excluded from the study (WHO, 2009).

3.3.5 Ethical Considerations

This study was approved by KEMRI/National Ethical Review Committee. Patient names were not used; instead, unique identification codes were used in order to ensure confidentiality. Written consent was sought from parents/guardians of the participants prior to sample collection. Information obtained from the patients was strictly confined to academic use only, unless otherwise there was clinical indication necessary to allow a shared confidentiality in good faith of the patient concerned.

3.4 Sample Collection

The stool samples were collected in clean sterile containers from children who met all the inclusion and none of the exclusion criteria hospitalized at either Kiambu District Hospital or Karuri Health Centre. Each sample was labeled according to the date of collection and the sample number. The samples were kept at 4^0C at the respective health facility awaiting transportation to the Nagasaki University, Institute of Tropical Medicine laboratories for processing.

3.5 Preparation of 10% Sample Suspension

10% fecal suspension was prepared forEnzyme Linked Immuno-sorbent Assay (ELISA) and RNA extraction.About 1g of stool sample or 100µl of swab suspension was added to 1ml of phosphate-buffer saline. The mixture was vortexed vigorously for 40 seconds followed bycentrifugation at 10,000 rpm for 5 minutes.All the supernatant (about 500µl) was transferred to new tubes and stored at -30°C until use.

3.6 Enzyme Linked Immuno-Sorbent Assay (ELISA) for Detection of Group A Rotavirus

Enzyme Linked Immuno-sorbent Assay (ELISA)was performed to screen for the presence of human serotype A rotavirus antigen in the 10% sample suspension. Briefly, 100µl of non-neutralizing monoclonal anti-human rotavirus antibody (Yo-156) directed against VP6, the group-specific antigen for all group A human rotaviruses, was coated on each plastic microtiter well as the capture antibody by an overnight incubation at 4°C. Unbound antibodies were washed away with 10mM PBS and each well blocked with 250µl of 1%BSA in PBST. 50µl of 10% sample suspension was added to each well, and the analyte in the sample would bind to the capture antibody on the solid phase during an overnight incubation at 4°C. Unbound components were washed away with 10mM PBST.50µl of anti-human rotavirus hyperimmune rabbit serum diluted 1:5000 with PBST containing 2.5% skim milk was then added to each well as the detector antibody followed by 1 hour incubation at 37°C. Unbound detecting antibody was washed away with 10mM PBST.50µl of peroxidase-conjugated donkey anti rabbit IgG (H+L chains) diluted 1:5000 with PBST (an enzyme-labeled antibody binding specifically to the

detection antibody) was added to each well followed by 1 hour incubation at 37°C. Unbound antibody was washed away with 10mM PBST. 100µl of O-phenylenediamine, a non-coloured substrate was added to each well, and the substrate would be converted to a coloured product by the enzyme bound to the antigen-antibody complex following 10-30 minute incubation at room temperature. Results were read spectrophotometrically at 490 nm with reference to 620 nm. Specimens with absorbance ≥0.3 were considered positive for group A human rotavirus whereas those with absorbance <0.3 were considered negative. KU strain (kind donation byFujita Health University, Department of Virology and Parasitology) was used as the positive control while phosphate buffered saline (PBS) was used as the negative control for this procedure.

3.7 Polyacrylamide Gel Electrophoresis (PAGE) for Determination of Rotavirus Electropherotypes

In order to determine the RNA migration patterns (electrophoretypes) of the segmented rotaviral genome and for confirmation of rotavirus ELISA results,polyacrylamide gel electrophoresis (PAGE)was carried out. Rotavirus double-stranded RNA was extracted from the ELISA positive 10% sample suspensions with ISOGEN-LS (Nippon Gene Co., Ltd., Toyama, Japan) according to the manufacturer's protocol. ISOGEN-LS is a complete and ready to use reagent for isolation of total RNA or for the simultaneous isolation of RNA, DNA and proteins from liquid samples of human, animal, plant, bacterial and viral origin. The composition of ISOGEN-LS includes phenol and guanidine thiocyanate in a mono-phase solution. In brief, 250µl of each of the 10% sample suspensions was homogenized in 750µl of ISOGEN-LS for 5 minutes at room temperature. The homogenate was separated into the aqueous and organic phases by the addition

of 200µl chloroform and subsequent centrifugation at 12,000 rpm for 15 minutes at 4°C. RNA would remain exclusively in the aqueous phase, DNA in the interphase, and proteins remain in the organic phase. RNA was then precipitated from the aqueous phase by addition of 200µl of isopropanol, and the resultant pellet washed with 1ml of 75% ethanol, briefly air-dried and finally solubilized in 50µl of double-distilled sterile water. The total RNA solution was electrophoresed on 10% polyacrylamide gel, 4.2 mm wide with 14 preformed wells for 1 hour 20 minutes at 35mA, 300V and 100W at room temperature. RNA segments were visualized by silver staining using EzStain Silver kit (ATTO Corporation, Japan) according to the manufacturer's protocol.

3.8 Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for VP4 and VP7 Genotyping

To determine the G and P genotypes of rotavirus strains present in the specimens and to confirm rotavirus ELISA results, a multiplexed semi-nested reverse transcription-polymerase chain reaction (RT-PCR)was carried out. In brief, 2µl of the total RNA solution from each sample was reverse transcribed into the complementary DNA (cDNA) on a thermocycler with a Ready-To-Go ReverTra Ace® qPCR RT Kit (Toyobo Co., Ltd., Japan) at the following temperatures for the following times: incubation at 42°C for 30 minutes;incubation at 99°C for 5 minutes; holding at 4°C for 5 minutes; and chilling on ice to primary PCR. The cDNA was then amplified in two steps, that is, primary PCR followed by nested PCR (Vera Gouvea *et al*., 1990).

In the first amplification, cDNAs corresponding to the full-length VP7 and VP4 genes were each amplified with a pair of primers for the 3' and 5' ends of each of the genes (Table 1). 25µl of

PCR reactions contained 2.5µl of KOD DNA polymerase reaction buffer, 2mM of each deoxynucleoside triphosphate, 0.4µM of each primer, 0.5µl of KOD-Plus-Ver.2 high fidelity DNA polymerase (TOYOBO Biotechnology Co. Ltd.) and 2µl of cDNA. PCR was performed on a thermocycler under the following conditions: 2 minutes at 95°C; 25 cycles of 30 seconds at 94°C, 30 seconds at 48°C and 1 minute at 72°C, and a final step of 7 minutes at 72°C followed by holding at 4°C. The PCR product was then subjected to 1% agarose gel electrophoresis at 100v for 35 minutes. Visualization of cDNA bands was achieved by staining the gels with ethidium bromide in Tris-Borate-Ethyldiaminetetraacetic acid(TBE) buffer solution for 20 minutes.

The second amplification of the primary PCR product of the VP7 gene was carried out using a mixture of primers that are specific to each of six variable regions of the VP7 genes of G1–4, G8, and G9 paired with a primer for the 3' end of the VP7 gene(Table 1). Similarly, the primary PCR product of the VP4 gene was amplified simultaneously using a mixture of primers that are specific to each of four variable regions of the VP4 genes of P1A[8], P1B[4], P2 and P3 paired with a primer for the 5' end of the VP4 gene(Table 1). PCR reactions contained 2.5µl of KOD DNA polymerase reaction buffer, 2mM of each deoxynucleoside triphosphate, 0.4µM of each primer, 0.5µl of KOD-Plus-Ver.2 high fidelity DNA polymerase (TOYOBO Biotechnology Co. Ltd.) and 2µl of primary PCR product. PCR was performed on a thermocycler under the same conditions as in the primary amplification. The PCR product was then subjected to 1% agarose gel electrophoresis at 100v for 35 minutes. Visualization of cDNA bands was achieved by staining the gels with ethidium bromide in TBE buffer solution for 20 minutes.

Table 1: Primer Sequences and their Location on the VP7 and VP4 Genesof Rotavirus (Gouvea*et al.*, 1990)

G-Genotype	Sequence (5'-3')	Product Length (base pairs)	Position on VP7 Gene (nt)
First PCR			
SA11	(-)GGTCACATCATACAATTCTAATCTAAG	1062	1039-1062
Wa	(+)GGCTTTAAAAGAGAGAATTTCCGTCTGG		1-28
Second PCR			
G1	(+)CAAGTACTCAAATCAATGATGG	749	314-335
G2	(+)GACTACAATGATATTACTAC	657	406-425
G3	(+)GACGCGACGTTGCAATTG	582	481-498
G4	(+)TCAAACGACAAATACAGCTA	394	669-688
G8	(+)GTCACACCATTTGTAAATTCG	885	178-198
G9	(+)CTAGATGTAACTACAACTAC	306	757-776

P-Genotype	Sequence (5'-3')	Product Length (base pairs)	Position on VP4 Gene (nt)
First PCR			
KU (P1A)	(+)TGGCTTCGTTCATTTATAGACA	1084	11-32
KU (P1A)	(-)CTAAATTGCTTTTGAATCATCCCA		1072-1094
Second PCR			
P1A	(-)ATATTCCTACGAGTTTAGTATC	497	487-508
P1B	(-)ACTAACATGTGGTTCAACTGCGAT	337	325-348
P2	(-)CTGAGCACGTTGATAAGTCAC	742	733-755
P3	(-)CGTATATTGATAGTTCATGGG	910	900-921

3.9 Data Analysis and Presentation

Raw data generated from this project was entered in Microsoft Excel Version 2010 with the password being protected. The data was analyzed using SPSSVersion 17.0. Findings were summarized into proportions/percentages, frequency tables, charts and graphs. The 95% confidence interval for prevalence was calculated using an online exact confidence interval for proportion method available at http://www.causascientia.org/math stat/ProportionCI.html.

CHAPTER FOUR

RESULTS

4.1 Demographic Characteristics of the Study Population

A total of 232 participants who met all the inclusion and none of the exclusion criteria were enrolled in this study and provided viable specimens for the study. Table 1 shows the demographic characteristics of the study population by sample type, gender, age and seasonal distributions. Most of the samples (94%) were stool type whereas 6% of them were swabs. This distribution was significantly different (χ^2 = 179.37; df = 1; P = 0.001). There was near equal distribution between male (56.7%) and female (43.5%) participants enrolled in this study. However the distribution between the gender was significantly different (χ^2 = 3.9; df = 1; P = 0.049). Majority (37.9%) of the participants were aged between 7 and 12 months followed by 22.7% of those agedbelow 6 months. Those aged from 19 to 24 months (6.9%) were the least (χ^2 = 58.9; df = 1; P = 0.001). Most (40.5%) of the samples were collected in the year 2009 while the least (10.3%) were collected in the year 2010 (χ^2 = 42.6; df = 3; P = 0.001).

Table 2: Demographic Characteristicsof the Type and Yearly Distribution of the Study Samples andGender and Age Distributionsof the Study Population, 2008-2011(n=232)

Characteristics	Frequency	Percentage	Chi-square	df	P Value
Sample type					
Stool	218	94	179.4	1	0.001
Swab	14	6			
Gender					
Male	131	56.5	3.9	1	0.049
Female	101	43.5			
Age Group					
>6 Months	52	22.4			
7 - 12 Months	87	37.5			
13 - 18 Months	37	15.9	58.9	4	0.001
19 - 24 Months	16	6.9			
>25 Months	40	17.2			
Year					
2008	54	23.3			
2009	94	40.5	42.6	3	0.001
2010	24	10.3			
2011	60	25.9			

NOTE: Df - Degree of freedom; P value - Level of significance

4.2. Prevalence of Rotavirus Infections

All the 232 specimens were screened for group A rotavirus using ELISA. Overall, 85 of the 232 had detectable rotavirus infection, representing a prevalence rate of 36.6% (95% CI 30.7 to 43.1) as shown in Figure 2.

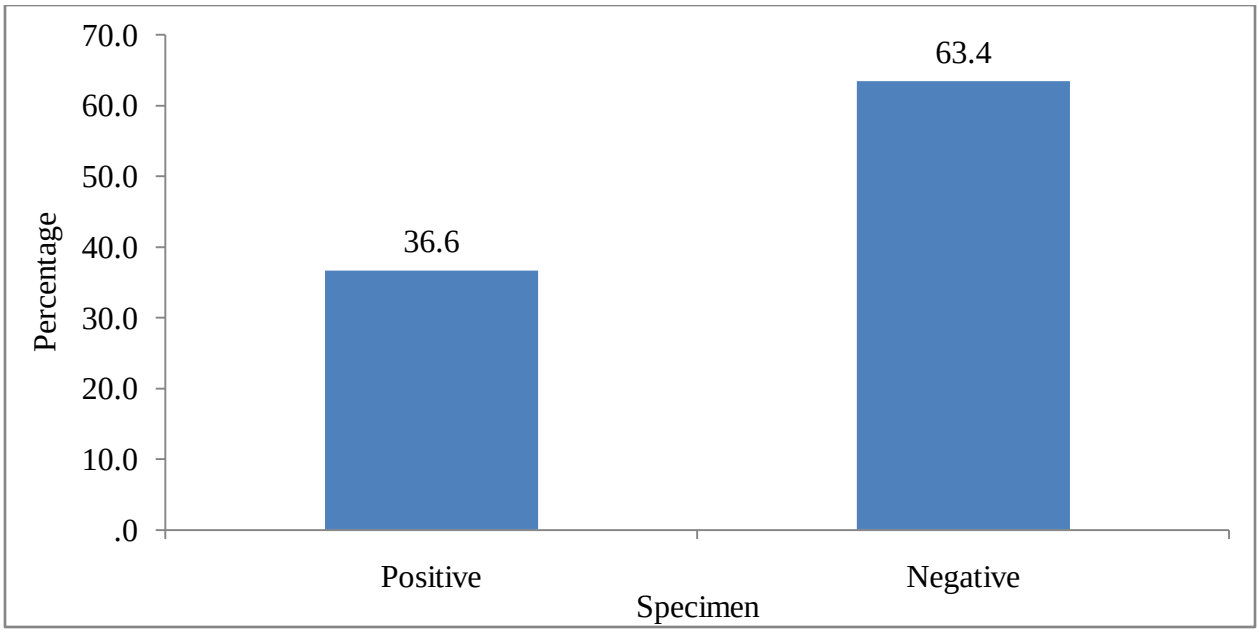

Figure 2: Prevalence of rotavirus infections by ELISAamong children aged below five years attending selected health facilities inKiambu District, Kenya between August 2008 and May 2011(n=232)

Of the 85 ELISA-positive specimens, 36.2% were from stool samples while 42.9% were detected from swabs samples.However, comparing the sample type and rotavirus detection showed no significant difference between specimen collection mode and the detection rates (χ^2 = 0.248; df = 1; P = 0.618). Out of the 85 rotavirus infections detected, majority (44.6%) were found in female while 30.5% of them were isolated from the male participants. The distribution of rotavirus infection by gender was significantly different (χ^2 = 4.829; df = 1; P = 0.028).However, the yearly distribution of rotavirus infections was not significantly different (χ^2 = 3438; df = 3; P = 0.329). 38.9%, 29.8%, 41.7%, and 43.3% of the infections was detected in the year 2008, 2009, 2010 and 2011, respectively (Table 3).

Table 3: Distribution of Rotavirus Infections by Type and Yearly Distribution of the Study Samples and Gender and Age Distributions of the Study Population, 2008-2011(n=232)

Characteristics	Distribution of Rotavirus Infections				Total	Chi-square	df	P - value
	Infected		Uninfected					
	Frequency	*Percentage*	*Frequency*	*Percentage*				
Specimen Type								
Stool	79	36.2	139	63.8	218	0.248	1	0.618
Swab	6	42.9	8	57.1	14			
Gender								
Male	40	30.5	91	69.5	131	4.829	1	0.028
Female	45	44.6	56	55.4	101			
Year								
2008	21	38.9	33	61.1	54			
2009	28	29.8	66	70.2	94	3.438	3	0.329
2010	10	41.7	14	58.3	24			
2011	26	43.3	34	56.7	60			
Age Group								
< 6 Months	17	32.7	35	76.3	52			
7 - 12 Months	43	49.4	44	50.6	87			
13 - 18 Months	13	35.1	24	64.9	37	12.162	4	0.016
19 - 24 Months	3	18.8	13	81.3	16			
>25 Months	9	22.5	31	77.5	40			
Total	**85**	**36.6%**	**147**	**63.4%**	**232**			

Df - Degree of freedom; P- value: Level of significance

4.3 Age Distribution of Rotavirus Infections

Rotavirus was detected most frequently in infants and young children of 2 years or less with a peak at 6 to 8 months of age. About 35% of these infections were identified among children aged 13 to 18 years while the least infection (18.8%) was found among infants aged 19 to 24 months. There was a reduction in the number of cases after two years of age (Figure 2). This distribution of infection by age was significant (χ^2 = 12.162; df = 4; P = 0.016).

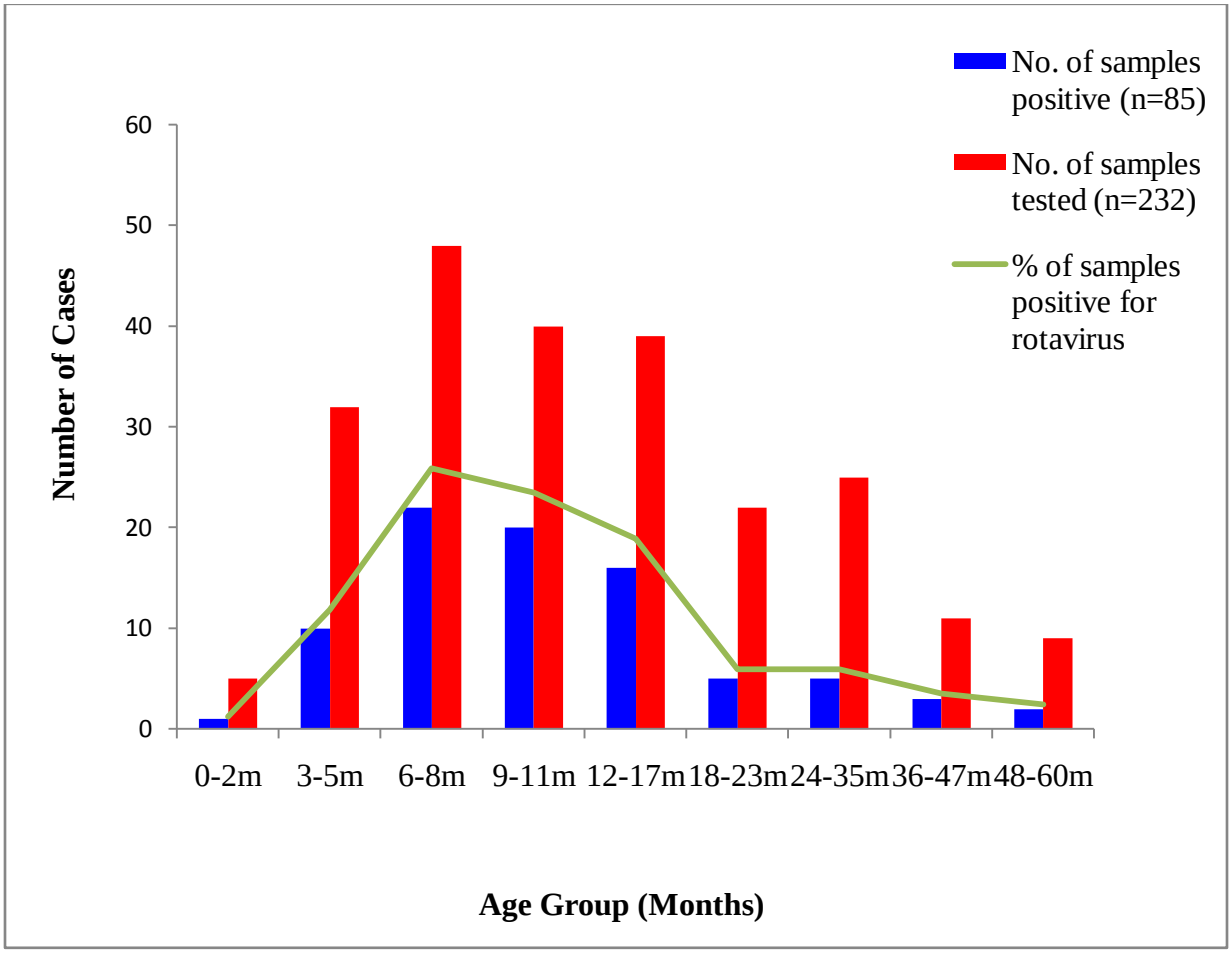

Figure 3: Variation by age of the study population (1-60 months) in the frequencies of rotavirus infections determined by ELISA for 232 fecal samples from children attending selected health facilities in Kiambu District, Kenya, 2008-2011.

4.4 Seasonality of Rotavirus Infections

4.4.1Seasonality of Rotavirus Infectionsin 2008

Rotavirus infections among children aged below five years attending selected health facilities in Kiambu District in the year 2008 varied significantly with meanmonthly temperature and precipitation recorded in Kiambu District in the year 2008 as shown in Table 4 ($\chi 2 = 10.793$; df = 4; P value = 0.029). Rotavirus infections exhibited a slight peak during the relatively cool and wet month of Septemberas shown in Table 4.

Table 4: Correlation of Monthly Rotavirus Infections Among Children Aged Below Five Years Attending Selected Health Facilities in Kiambu District, Kenya in 2008withMean Monthly Temperature and Precipitation Recorded in Kiambu District in 2008. Climatic Data Used with Permission from the Kenya Meteorological Department

			2008 Rotavirus infection				
Month	Parameter	Total	Positive	Negative	Chi-square	df	P value
	Temperature						
August	17.15	8	2	6			
September	18.3	14	10	4			
October	19.4	14	5	9			
November	22	5	0	5	10.793	4	0.029
December	23	11	3	8			
	Total	52	20	32			
	Rainfall						
August	2.7	8	2	6			
December	3.8	11	3	8			
October	22.5	14	5	9	10.793	4	0.029
September	36.2	14	10	4			
November	62.5	5	0	5			
	Total	52	20	32			

4.4.2Seasonality of Rotavirus Infectionsin 2009

Similar to the year 2008, rotavirus infections among children aged below five years attending selected health facilities in Kiambu District in the year 2009 varied significantly with meanmonthly temperature and precipitation recorded in Kiambu District in the year 2009 as shown in Table 5($\chi 2$ = 16.175; df = 8; P value = 0.04). Rotavirus infections exhibited a slight peak during the relatively cool and wet months as shown in Table 5 below.

Table 5: Correlation of Monthly Rotavirus Infections Among Children Aged Below Five Years Attending Selected Health Facilities in Kiambu District, Kenya in 2009withMean Monthly Temperature and Precipitation Recorded in Kiambu District in 2009. Climatic Data Used with Permission from the Kenya Meteorological Department

		2009 Rotavirus infection					
Month	Parameter	Total	Positive	Negative	Chi-square	df	P value
	Temperature						
June	18.05	12	1	11			
May	18.9	14	4	10			
September	18.95	1	1	0			
December	19.2	1	0	1	16.175	8	0.04
January	19.3	9	2	7			
February	19.6	25	10	15			
April	19.95	10	0	10			
March	20.75	22	11	11			
July	26.75	2	0	2			
	Total	96	29	67			
	Rainfall						
September	4.1	1	1	0			
July	6.8	2	0	2			
February	8.5	25	10	15			
June	15.8	12	1	11			
April	16.8	10	0	10	16.175	8	0.04
January	25.7	9	2	7			
March	33.3	22	11	11			
December	46.6	1	0	1			
May	57.2	14	4	10			
	Total	96	29	67			

4.4.3Seasonality of Rotavirus Infectionsin 2010

Rotavirus was scantly isolated in the year 2010 andinfections among children aged below five years attending selected health facilities in Kiambu District in the year 2010 did not vary significantly with meanmonthly temperature and precipitation recorded in Kiambu District in the same year, as shown in Table 6 ($\chi 2 = 2.511$; df = 3; P value = 0.473).

Table 6: Correlation of Monthly Rotavirus Infections Among Children Aged Below Five Years Attending Selected Health Facilities in Kiambu District, Kenya in 2010withMean Monthly Temperature and Precipitation Recorded in Kiambu District in 2010. Climatic Data Used with Permission from the Kenya Meteorological Department

		2010 Rotavirus infection					
Month	Parameter	Total	Positive	Negative	Chi-square	df	P value
	Temperature						
September	17.9	5	1	4			
November	18.45	14	5	9			
October	19.3	9	5	4	2.511	3	0.473
March	19.35	1	0	1			
	Total	**29**	**11**	**18**			
	Rainfall						
September	19.9	5	1	4			
October	64.3	9	5	4			
November	93.3	14	5	9	2.511	3	0.473
March	250.3	1	0	1			
	Total	**29**	**11**	**18**			

4.4.3Seasonality of Rotavirus Infections in 2011

Similar to the year 2010, rotavirusinfections among children aged below five years attending selected health facilities in Kiambu District in the year 2011 did not vary significantly with meanmonthly temperature and precipitation recorded in Kiambu District in the same year, as shown in Table 7($\chi 2$ = 1.867; df =23; P value = 0.393).

Table 7: Correlation of Monthly Rotavirus Infections Among Children Aged Below Five Years Attending Selected Health Facilities in Kiambu District, Kenya in 2011withMean Monthly Temperature and Precipitation Recorded in Kiambu District in 2011. Climatic Data Used with Permission from the Kenya Meteorological Department

			2011 Rotavirus infection				
Month	Parameter	Total	Positive	Negative	Chi-square	df	P value
	Temperature						
May	19	7	3	4			
April	19.65	29	11	18			
March	20.15	19	11	8	1.867	2	0.393
	Total	55	25	30			
	Rainfall						
April	80.7	29	11	18			
May	93.7	7	3	4	1.867	2	0.393
March	147.7	19	11	8			
	Total	55	25	30			

4.4.4 Overall Seasonality of Rotavirus Infections

Overall, rotavirus infections wererecorded year-round in this study with slight peaks during the months of March and September (Figure 4).Attempts to univariatelyrelate these seasonal patterns of rotavirus infections to climatic variables such as temperature and rainfall established no statistically significant association ($\chi 2 = 96$; df =90; P value = 0.313).

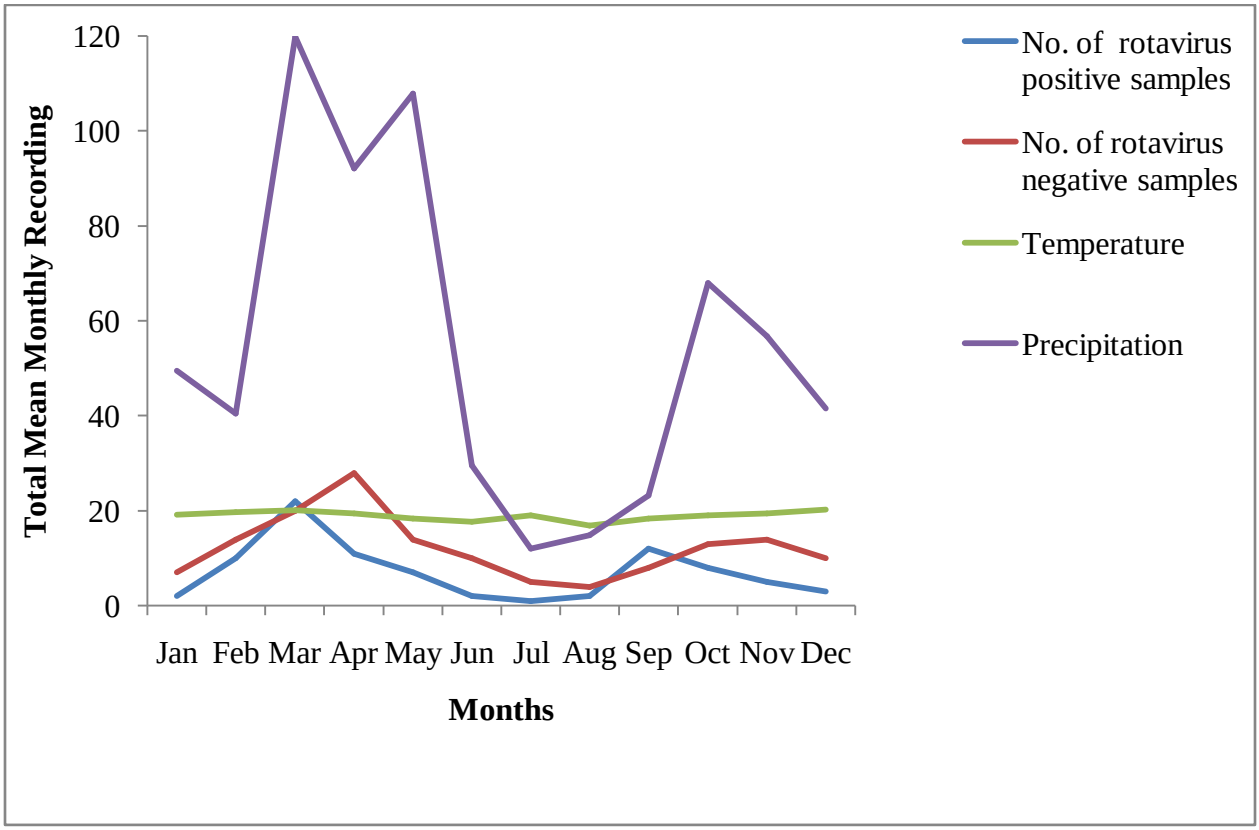

Figure 4: Correlation between the monthly rotavirus cases among children attending selected health facilities in Kiambu District, Kenya, 2008-2011and total mean monthly rainfall and temperature recorded in all the years in Kiambu District.Climatic data used with permission from the Kenya Meteorological Department.

4.5Rotavirus Electropherotypes

Total RNA extracted from a total of 85 rotavirus-positive specimens was subjected to PAGE and the dsRNA migration patterns (electropherotypes) were obtained as shown in Figure 5.Rotaviruses whose RNA profiles had fast migrating RNA segments 10 and l1 were labeled ‘long’ electropherotypes(Figure 5; lanes 2, 3, 5, 6, 8, 10&12), while those with RNA profiles characterized by slower migrating segments 10 and 11 were labeled 'short' electropherotypes (Figure 5; lane 1). Minor differences in RNA profile within each distinct electropherotype were also observed(Figure 5; lanes 8, 10 and 12).

All electropherotypes were group A rotaviruses based on the characteristic 4-2-3-2 distribution pattern of the genomic RNA segments. Out of the 85 ELISA-positive samples, 58 (68.1%) gave visible RNA profileswhereas 28 (32.9%) gave invisible profile. Of the visible RNA profiles, majority (92.9%)were long electropherotypes. Short electropherotypesaccounted for 5.3% with 1.8% specimens exhibiting more than 11 RNA segments, suggesting the occurrence of mixed infection and/or RNA rearrangement ($\chi^2 = 344.621$; df = 1; P = 0.001).

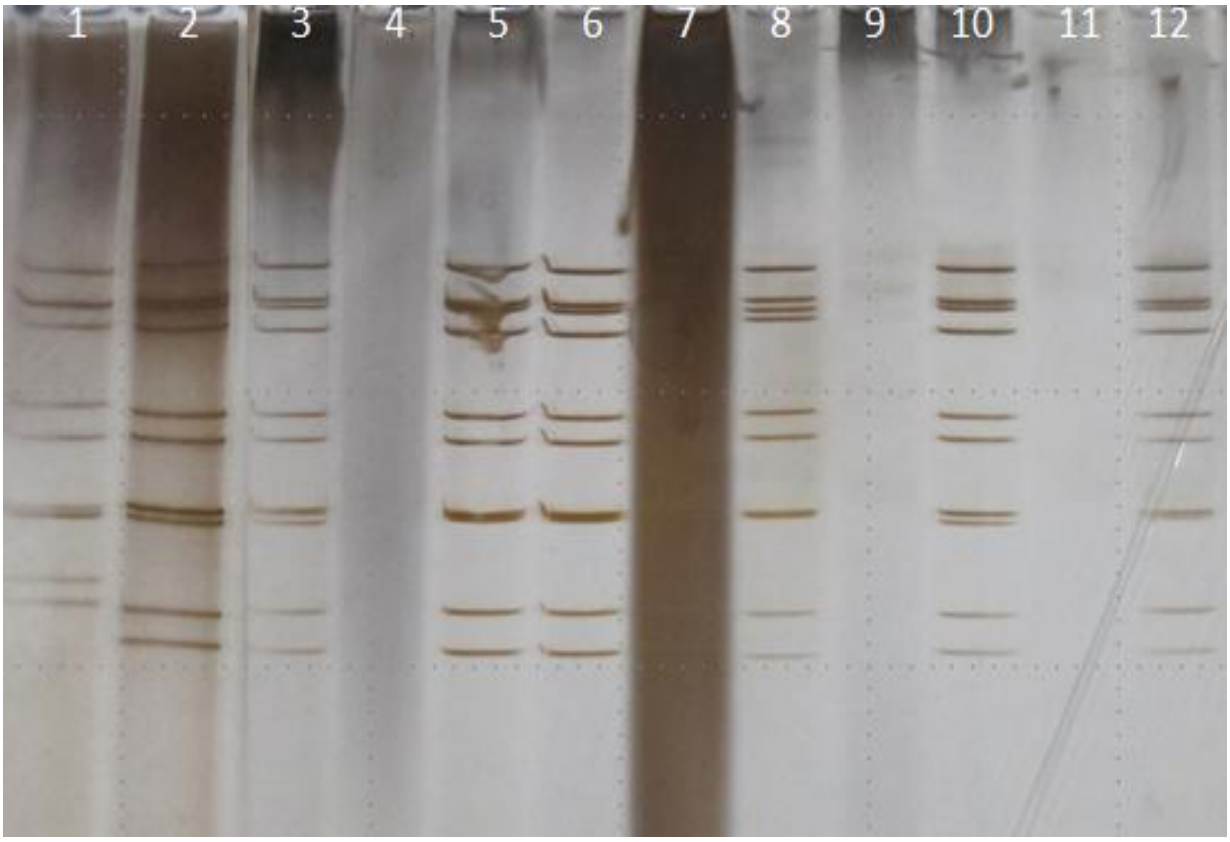

Figure 5: Pattern variability of rotavirus electropherotypes on 10% polyacrylamide gel,determined for 85 ELISA-positive samples from children attending selected health facilities in Kiambu District, Kenya, 2008-2011. Lane 1:Short electropherotype; Lanes 2, 3, 5, 6, 8, 10&12: Long chain electropherotypes,exhibiting minor differences in RNA profile within theelectropherotype;Lanes 4, 7, 9&11: Invisible electropherotypes.

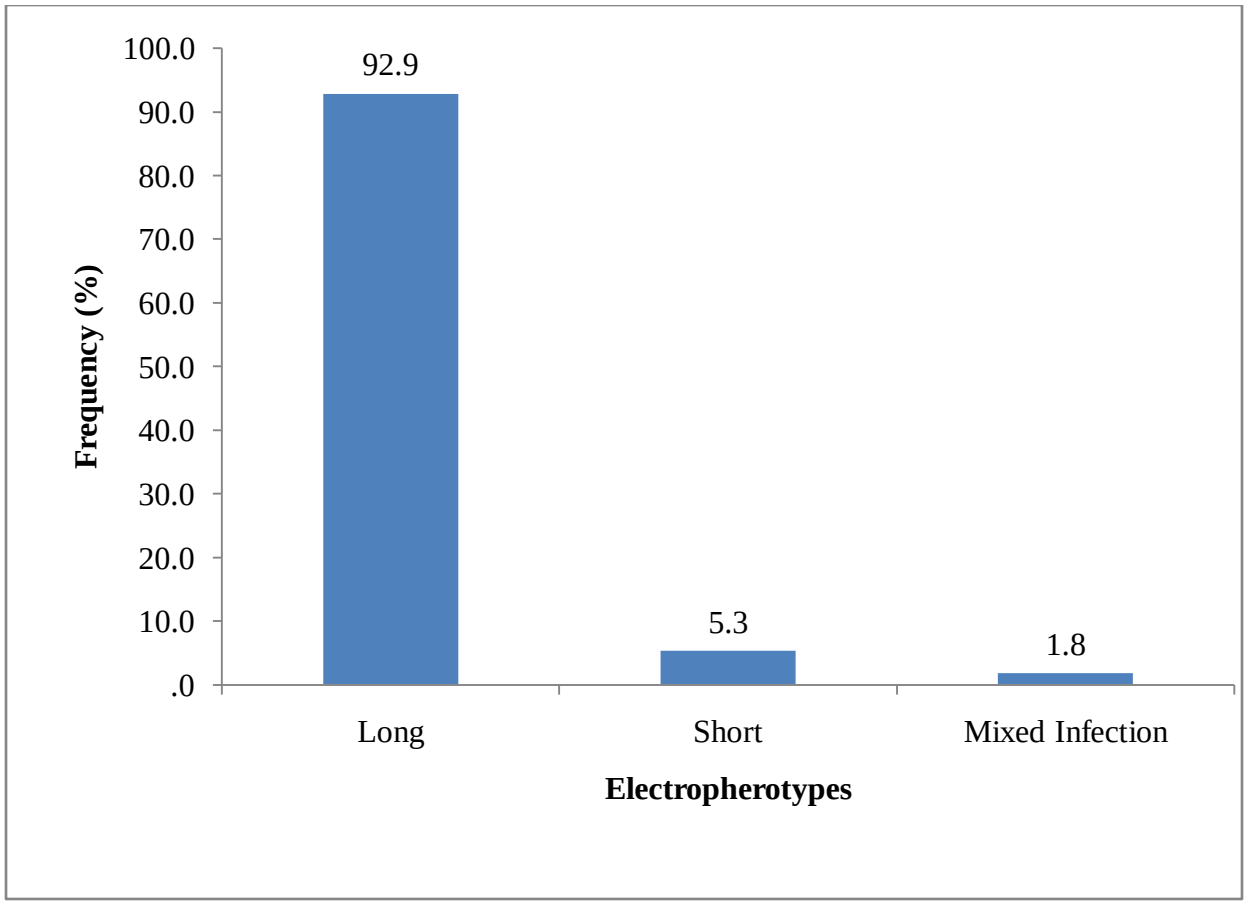

Figure 6: Distribution of visible electrophoretic patternsof rotavirus strains on 10% polyacrylamide gel,determined for 58 of the 85 ELISA-positive samples from children attending selected health facilities in Kiambu District, Kenya, 2008-2011.

4.6 Rotavirus Genetic Diversity by Multiplexed Semi-Nested RT-PCR

4.6.1 Primary PCR

The cDNAs corresponding to the full-length VP7 and VP4 genes were detected in 55 of 85 (64.7%) rotavirus-positive specimens following amplification with a pair of primers for the 3' and 5' ends of each of the genes as shown in Figure 6.

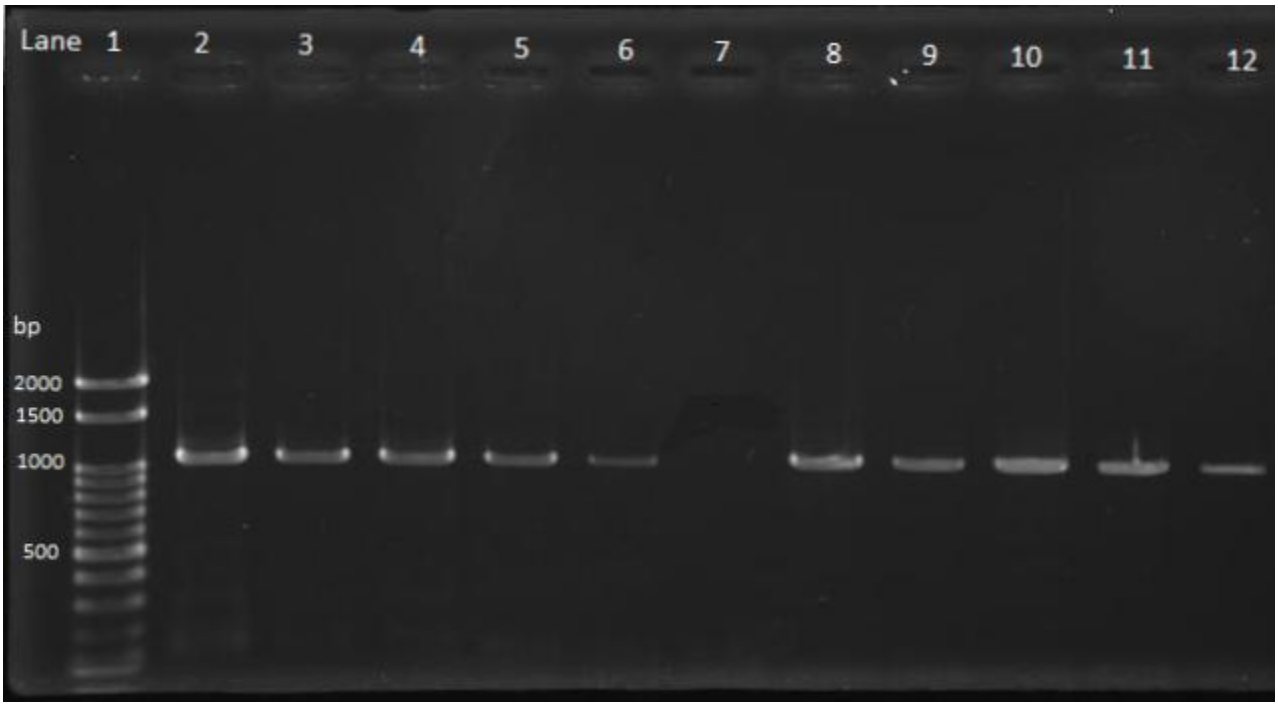

Figure 7: cDNAbands corresponding to the rotavirus VP7 and VP4 genes as detected on 1% agarose gel following primary PCRof the 85 ELISA-positive samples from children attending selected health facilities in Kiambu District, Kenya, 2008-2011. Lane 1: 100bp DNA marker; Lanes 2&8:VP7 and VP4 genes of the KU strain (positive control), respectively; Lanes3-6: Amplified VP7 gene (expected band size, 1062 bp); Lane 7: Negative Control; Lanes 9-12: Amplified VP4 gene (expected band size, 1084 bp).

4.6.2 VP7 Genotyping

Rotavirus G genotypes could be determined in 55 of 85 specimens. Genotype G1 was predominant in the specimens analyzed and constituted 44.7% of the strains. In addition, other usual global genotypes, including G2, G4 and G9 were detected in10.6%, 4.7% and 1.2% of specimens, respectively. G8, the African-specific strain was detected at 8.2%. In 30.6% of the ELISA-positive specimens, G genotypes could not be detected using the existing primer set (χ^2 = 447.48; df = 1; P = 0.001) as shown in Figure 9.

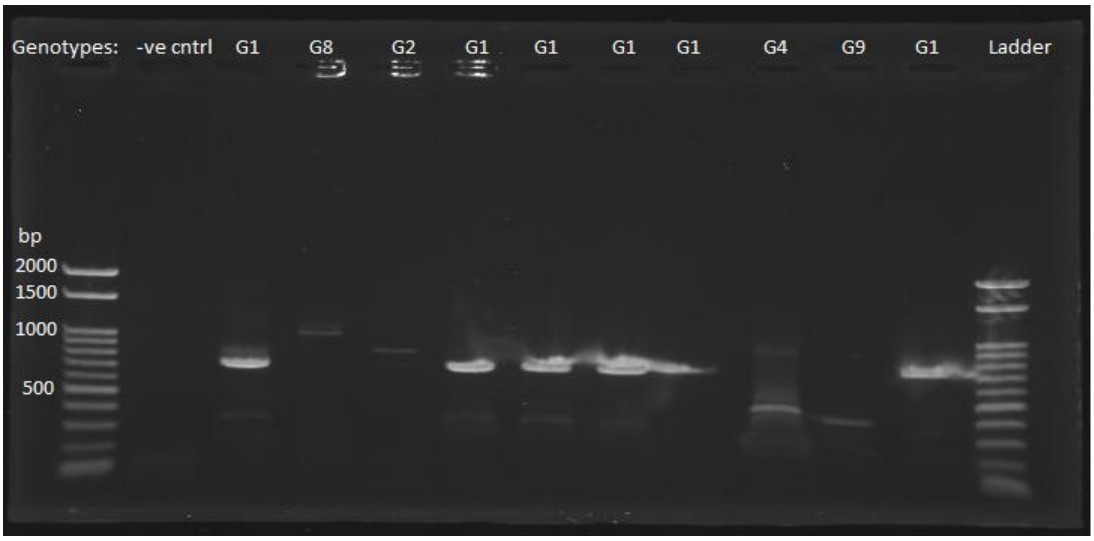

Figure 8: Bands corresponding to different rotavirus G genotypes as detected on 1% agarose gel following multiplexed semi-nested RT-PCR of the 85 ELISA-positive samples from children attending selected health facilities in Kiambu District, Kenya, 2008-2011. Lanes 1&13: 100bp DNA marker;Lane 2: Negative Control; Lane 3: KU strain (positive control); Lanes6, 7, 8, 9&12: G1 (expected band size, 749 bp); Lane 4: G8 (expected band size, 885 bp); Lane 10: G4 (expected band size, 394 bp); Lane 11: G9 (expected band size, 306 bp).

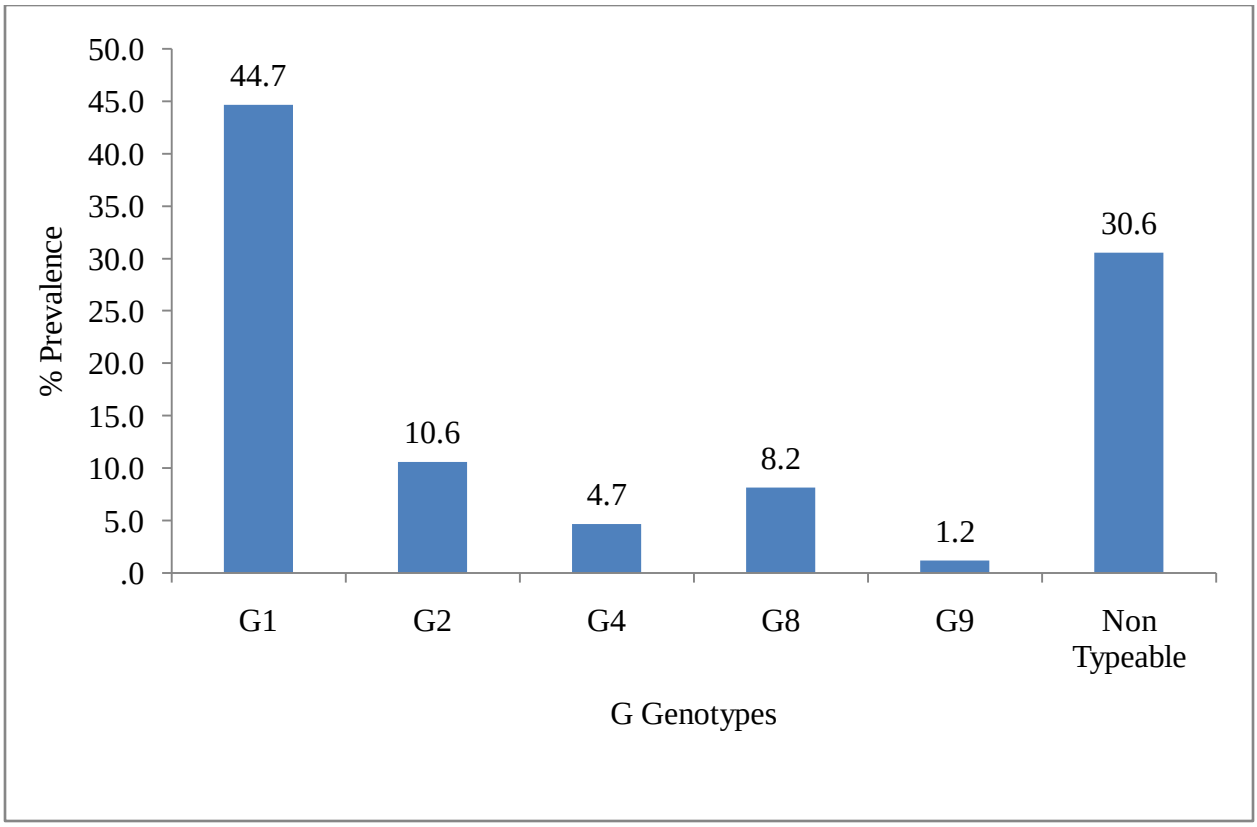

Figure 9: The distribution of rotavirus G genotypesdetermined by multiplexed semi-nested RT-PCR for the 85 ELISA-positive samples from children attending selected health facilities in Kiambu District, Kenya, 2008-2011. Non-typeable refers to those strains which could not be detected using the existing primer set.

4.6.3 VP4 Genotyping

Figure 9 shows various rotavirus P genotypes isolated during this study. Out of the 85 specimens analyzed, genotypes P1A [8] and P1B [4] predominated at 28.2% and 25.9% respectively. Genotype P2[6], the African-specific strain was isolated at 1.2%. In 49.4% of theELISA-positive specimens, P genotypes could not be detected using the existing primer set (χ^2 = 376.379; df = 1; P = 0.001).

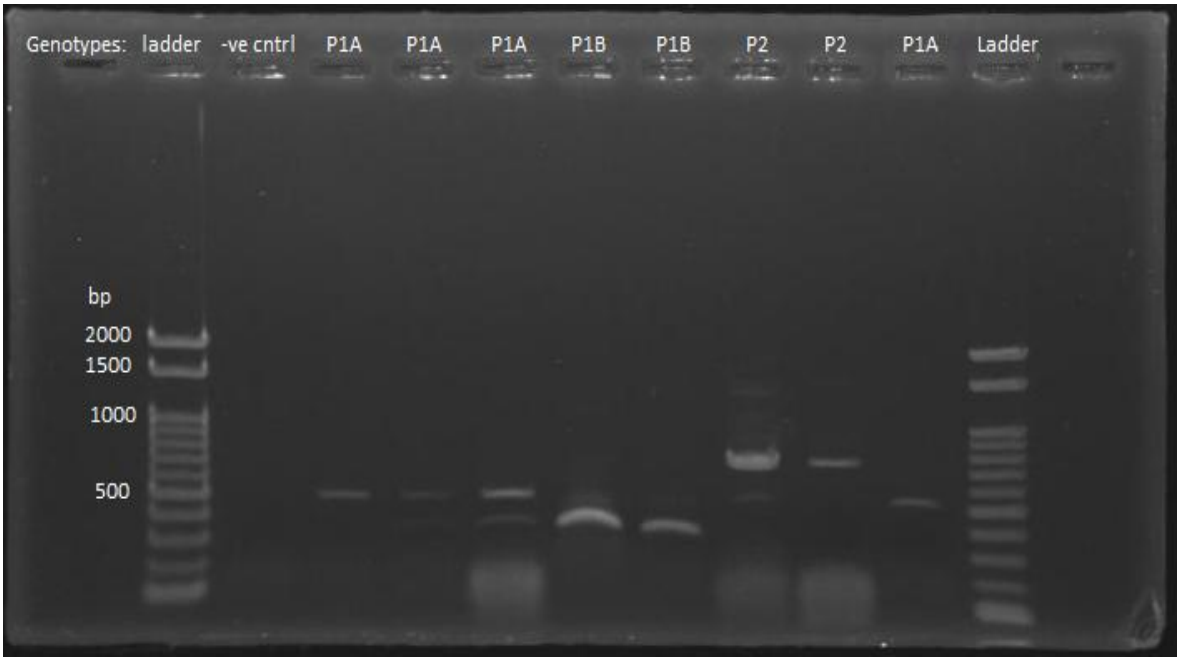

Figure 10: Bands corresponding to different rotavirus P genotypes as detected on 1% agarose gel following multiplexed semi-nested RT-PCR of the 85 ELISA-positive samples from children attending selected health facilities in Kiambu District, Kenya, 2008-2011. Lanes 2&12: 100bp DNA marker;Lane 3: Negative Control; Lane 4: KU strain (positive control); Lanes5, 6&11: P1A[8] (expected band size, 497 bp); Lanes7&8: P1B[4] (expected band size, 337 bp); Lanes 9& 10: P2[6] (expected band size, 742 bp).

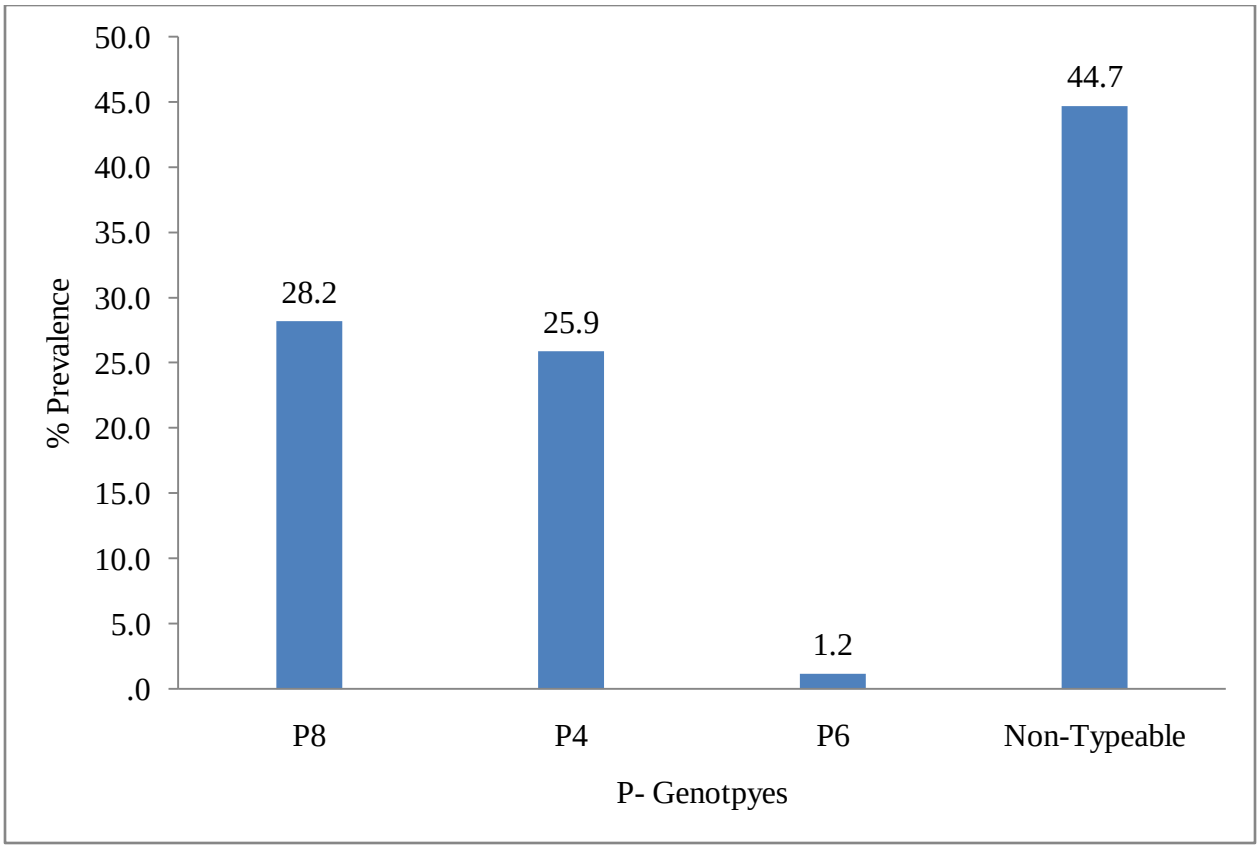

Figure 11: The distribution of rotavirus P genotypesdetermined by multiplexed semi-nested RT-PCR for the 85 ELISA-positive samples from children attending selected health facilities in Kiambu District, Kenya, 2008-2011. Non-typeable refers to those strains which could not be detected using the existing primer set.

4.7Rotavirus G and P Genotype Combinations

Table 8 shows the different rotavirus strains detected in this study. There were 10 different G and P type combinations detected in ELISA-positive specimens. G1P[8] constitutedthe majority of the rotavirus strainsat 21.2%, followed by G1P[4] and G2P[4] at 9.4% and 7.1% respectively. Other strains included; 5.9% G4P[4], 3.5%G2P[4], 2.6% G4P[8] and G4P[4], 1.2% G8P[8] and G1P2[6]. 12.9% of G1 and 1.2% of G8 associated with P types that could not be typed using the primer set. 30.6% of the ELISA-positive samples could not be typed for both G and P genotypes using the primer sets (χ^2 = 376.379; df = 1; P = 0.001).

Table 8: Rotavirus P-G Type Combinations in 85 Infections among Children Aged Below Five Years Attending Selected Health Facilities in Kiambu District, Kenya, 2008-2011

Strain Type	Frequency	Percentage	Chi-square	df	P Value
G1P[8]	18	21.2			
G2P[8]	3	3.5			
G4P[8]	2	2.6			
G8P[8]	1	1.2			
G1P[4]	8	9.4			
G2P[4]	6	7.1	1346.82	11	0.001
G4P[4]	2	2.6			
G8P[4]	5	5.9			
G1P[2]	1	1.2			
G9P[4]	1	1.2			
G1P[NT]	11	12.9			
G8P[NT]	1	1.2			
P[NT]G[NT]	26	30.6			
Total	**85**	**100**			

NOTE: Df - Degree of freedom; P value - Level of significance; NT-nontypeable

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Prevalence of Rotavirus Infections

In this study conducted between August 2008 and June 2011, 232 stool specimens were collected from infants and young children below 5 years of age with acute gastroenteritis hospitalized at Kiambu District Hospital andKaruri Health Centre in Kiambu district, Kenya. Rotavirus was detected in over 36.6% of all these cases (85/232). The results from this study reveal a significant increase in the overall prevalence of rotavirus infection over the years in Kenya (Gatheru *et al.,* 1993; Nakata *et al.,* 1999; Kiulia *et al.,* 2008).

The high infection rate of rotavirus may be associated with the dual condition of extremely high virus concentration in faeces (more than 10^9 virus particles/g)of symptomatic and asymptomatic individuals and the low inoculums(10–100 virus particles) required for infection(Grassi *et al.,* 2009). Similarly, several authors have suggested that the ability of rotavirus to remain viable on inanimate surfaces for several days when dried from a fecal suspension provide indirect evidence to show that fomites and environmental surfaces possess a strong potential for spreading rotavirus gastroenteritis (Ansari *et al.,* 1991; Haffejee, 1995; Torok*et al.,* 1997). In addition, widespread viral contamination of water bodies and prolonged persistence of infective virus in ground and surface water may also be contributing to the high prevalence rate of rotavirus infection observed in this study (Espinosa *et al.,* 2008).

5.2 Age Distribution of Rotavirus Infections

Rotavirus infection occurred in all age groups between 1 month and 5 years with a peak at 6 to 8 months of age. There was a low rate of rotavirus isolation among the infants aged below 6 months and a drastic reduction in the number of cases among the children above 2 years of age. This phenomenon may be attributed to the fact that infants are usually protected against rotavirus infection by the high levels of transplacental antibodies acquired from the mother, immune and non-immune components of breast milk, and the large amount of gastric acid in the infant's digestive tract.However, the maternal antibodies are only protective in the first few months of infant life owing to their short half-life (3-4 weeks). As a result, this protection is lost as infants approach 6 months when the antibodies wane, hence the increase in the number of cases of rotavirus gastroenteritis as from 6 months (Patel *et al.*, 2009). Nonetheless, it was quite surprising in this study to isolate rotavirus in a one month old infant.

Breast milk, on the other hand, contains both IgA antibodies that can neutralize rotavirus and receptor analogues (lactadherin) that can adsorb to the virus and inhibit its attachment (Newburg *et al.,* 1998). These factors, like transplacental antibodies, are greatest during the first days after parturition and decrease as breastfeeding progresses (Kent *et al.,* 2006). An early study of the neutralization of a serotype 1 virus, strain Wa, in breast milk samples collected from nursing mothers in Bangladesh yielded results that may be instructive (Glass and Stoll, 1989). During this*in vitro* study, breast milk was mixed with an equal volume of live virus.About30% of the breast milk samples were able to neutralize 99% (2 logs) of virus, and 60% of the breast milk

samples could neutralize 90% (1 log) of virus. Other factors in breast milk might entrap virus and prevent it from attachment.

The low isolation of rotavirus among the infants may also be contributed by the damage on rotavirus by the low pH caused by the large amount of stomach acid present in the infants' digestive tract (Ing*et al.*, 1991). Similarly, the reduced frequency of rotavirus infection after two years of age may be attributed to the fact that most children from this age would have suffered from at least one episode of rotavirus gastroenteritis and this confers immunity to subsequent infections(Vela´squez*et al.*, 1996). Each additional infection expands the population of B cells producing cross-reactive antibodies that can recognize multiple serotypes rendering repeat infections less severe and thereby requiring little or no admission to health facilities (Fischer *et al.*, 2002).

5.3 Seasonality of Rotavirus Infections

Rotavirus was found year-round in this study with slight peaks and valleys in some months. This observation is consistent withrecent country-level assessments of rotavirus epidemiology conducted in various tropical countries in anticipation of rotavirus vaccination programmes(Levy *et al.*, 2009). The assessments have noted seasonal peaks to occur year-round in different tropical countries and can vary over time in the same country. However, the effect of seasonal changes on rotavirus incidence observed here is not as extreme as it isseen in temperate areas of the world where the incidence often goes to zero in some months(Cook *et al.*, 1990). One explanation for this phenomenon is that less climatic variability exists in tropical climates and zones, so

variations in climatological variables are not large enough to cause the observed effect(Atchison *et al.,* 2009; D'Souza *et al.,* 2008).

Similar to other related studies reviewed by Levy and colleagues (2009), attempts to relate the seasonal patterns observed in this study to climatic variables such as temperature and rainfall led to conflicting results; it is possible the effect of certain climatic variables is context specific. In the univariate analyses, a statistically significant positive association between monthly rotavirus incidence and mean monthly temperature and rainfall was found in the year 2008 and 2009. Conversely, monthly rotavirus incidence in the year 2010 and 2011showed no correlation with the climatological variables. Further univariate analyses of the correlation between the monthly rotavirus cases and grand mean monthly rainfall and temperature recorded in all the years showed no statistically significant association. The heterogeneity in effect observed in this studymight be due to the fact the analyses did not take into account additional factors potentially affecting rotavirus transmission, such as sanitation and hygiene practices or flood peaks.

Thus, understanding the transmission of rotavirus might shed more light on the seasonal patterns of rotavirus infections.For instance, several authors of the articles reviewed by Levy and colleagues (2009) noted multiple peaks in rotavirus incidence as affected by the monsoon rains. Flooding in conjunction with poor sanitation could augment the waterborne component of rotavirus transmission, obfuscating the seasonal patterns, which might be driven more by other routes of transmission, such as the air or fomites.Strong evidence suggests that rotavirus is a waterborne pathogen. The virus can retain its infectivity for several days in aqueous environments, and waterborne spread has been implicated in a number of rotavirus outbreaks

(Ansari *et al.*, 1991). However, the high rates of infection in the first three years of life regardless of sanitary conditions, the failure to document fecal-oral transmission in several outbreaks of rotavirus diarrhea, and the dramatic spread of rotavirus over large geographic areas in the winter in temperate zones suggests that water alone may not be responsible for all rotavirus transmission.

Many authors have also suggested that rotavirus spreads through the air (Hashizume*et al.*, 2008).Bishop (1996) proposes that such transmission might lie in the airborne spread of aerosolized particles that are ingested, rather than through respiratory tract infections.A relative rise in temperatures might increase the aerial transport of dried, contaminated faecal material (in the form of droplet nuclei), and might also lead to increased formation of dust, which could provide a substrate for the virus particles (Ansari *et al.*, 1991). Airborne particles could settle out and infect water supplies or environmental surfaces, or could be ingested(Haffejee, 1995). Some mechanical force would likely be required for aerosolization to occur, and wind might play this role, as well as help disperse the particles once formed. In a four-year study of rotavirus in Pune, India, a tight correlation was seen between number of days with easterly wind and the number of rotavirus diarrhea cases as functions of time (Purohit*et al.*, 1998). Further research on the relationship between wind patterns and rotavirus incidence might shed further light on this potential transmission mechanism.

In conclusion, the fact that rotavirus persists year-roundwith slight peaks and valleys in some months in tropical areas of the world, as observed in this and other studies, and that rotavirus responds to climatic changes in many different climatic zones throughout the world, may

suggestthat it is not an absolute temperature, humidity or rainfall level that rotavirus prefers, but rather a relative change in climatic conditions (Atchison *et al.,* 2009; D'Souza *et al.,* 2008). Such environmental factors probably play a role in organizing the peak of rotavirus transmission to times when conditions are most favourable to virus survivability and transmission. These results therefore suggest that paying close attention to local climatic conditions will improve our understanding of the transmission and epidemiology of rotavirus disease.

5.4Rotavirus Electropherotypes

Besides serological classifications, the genome of rotavirus which consists of 11 segments of dsRNA can be separated into distinct bands by PAGE and visualized by comassine blue or silver staining. The RNA profile (long or short electropherotype) obtained is based on the migration rate of gene 11. The short electropherotype phenotype results from a partial duplication in gene 11, which causes it to migrate more slowly than gene segment 10. The standard-sized gene 11 of long electropherotype strains migrates faster than segment 10. Each distinct dsRNA profile defines a different viral electropherotype or strain (Fischer and Gentsch, 2004).

In this study, bands characteristic of rotavirus double-stranded RNA were detected by PAGE in 58 of the 85(68.2%) samples in which the presence of rotavirus had been demonstrated by ELISA. All of the visible electropherotypes exhibited the characteristic 4-2-3-2 distribution pattern of the 11 segments of dsRNAs thereby leading to a conclusion that they were all group A rotaviruses. Consistent with other Kenyan studies, strains isolated in this study displayed great genetic diversity, with 4 long and 2 short electropherotypes (Nyangao*et al.,* 2010). One

electropherotype predominated during the study period. A basic variance in RNA profiles among the electropherotypes was the separate migration of all 11 RNA segments or co-migration of certain RNA segments (Figure 8). The former was more common among the long electropherotypes whereas all short electropherotypes had the latter profile with co-migration of segments 2 and 3.Besides this basic difference, there were other distinctive differences among different electropherotypes. In long electropherotypes, segments 2 and 3 of the large RNA segments 1-4 varied the most in relative migration positions that ranged from far apart to together. In contrast, segments l and 4 had basically the same mobility, or position in the gel. The mobility of segments 5 and 6 was essentially similar in all long electropherotypes. The closely running triplet of segments 7, 8 and 9 had the most number of variable patterns: the different positions of segment 8 relative to 7 produced migration patterns in which segment 8 was located equidistant to segments 7 and 9 and progressively closer to segment 7 until allco-migrated. Segments 10 and 11 occupied rather similar positions in all electropherotypes.

Among short electropherotypes, the mobility of RNA segments 1 to 4 was similar in allthe electropherotypes. Segments 5 and 6 had2different migration patterns. The migration patterns of the triplet segments 7, 8 and 9, and the small segments 10 and l1 were different in all theelectropherotypes. In stool sample of one infant more than 11 RNA segments were found, suggesting the possibility of simultaneous infection by more than one electropherotype and/or the occurrence of modification in the length of RNA segments during infection. The implication of such mixed infection is that reassortant strains are likely to emerge following the simultaneous infection of an individual with different isolates (Steele *et al.,* 1988).The absence of the band in

some ELISA-positive specimens could be due to too little RNA or its destruction during extraction by phenol/chloroform (Pennap*et al.,* 2000).

Electropherotypes are not used in formal virus taxonomy to classify rotaviruses into different serotypes and subgroups because corresponding segments of rotaviruses with different genome composition can co-migrate, and co-migrating RNA segments of different electropherotypes can code for proteins with different serotypic specificities (Beards, 1982). Nevertheless, electropherotypes are useful epidemiological indicators on the evolution and spread of rotavirus strains, and the observation of changes in electropherotypes helps to monitor the patterns of disease outbreaks and transmissions(Matthijnssens *et al.,* 2008). Since the overall electropherotypic pattern produced is a reproducible trait of a virus strain, the heterogeneity of electropherotypes observed in this study provides vital information on genetic diversity of rotavirus strains circulating in Kiambu district and may be useful in tracing spread through the population.

5.5 VP4 and VP7 Genotyping

In this study conducted between August 2008 and May 2011, there were 10 different G and P type combinations detected in rotavirus-positive specimens. These results are consistent with those of other African studies describing various P specificities associated G genotypes (Page *etal.,* 2010; Esona*et al.,* 2010).Serotype G1P[8] strains were predominant in the specimens analyzed, followed by G1P[4], G2P[4], and G8P[4] strains. These serotypes, including G3P[8] and G9P[8] are thought to be an important cause of diarrhea in infants and young children

worldwide, representing almost 72% of all strains detected (Gentsch*et al*., 2005). However, the prevalence of these strains can differ markedly in various settings and from one year to the next. For instance, serotype G3 strains predominantly circulating in the years 1999 and 2000 (Kiulia*et al.,* 2008)were replaced by G1 strains and were not isolated during this study period of the year 2008 to 2011. Only 4 G4 strains were detected in this study period in the specimens analyzed. This is not unusual, however, because the circulation of G4 strains occurs in sporadic epidemics (Bishop *et al.,* 2001). The prevalence of G4 strains decreased from 41.6% (predominant) in 1989–1991 to 8% in 2000-2002 and 4.7% in this study period, and these strains seem to be disappearing from circulation in Kenya (Kiulia*et al.,* 2008). Similarly, G9 strains detected only in Kenya in 1999 (Steele and Ivanoff, 2003) and which predominated in 2004-2005 study (Kiulia*et al.,* 2006) could be isolated from only one specimen in this study, indicating its drastic decline in prevalence over the years. Serotype G1 and G8 strains were associated with a variety of VP4 P types: P[8], P[4], and P[2]. These results are consistent with those of other African studies describing various P specificities associated with G1 and G8 strains (Page and Steel, 2004). Whereas serotype G8 strains were detected in Nairobi in the early 1990s (Nakata *et al.,* 1999), serotype G1 strains were detected predominantly in Kenya in 1982-1983, 1989-1991 and 2000-2002study periods (Kiulia*et al.,* 2008). This study shows that G1 and G8 strains are continuously circulating in the Kenyan population. Kenyan G2P[4] strains have previously been shown to display diversity and cluster separately during phylogenetic analyses (Page and Steel, 2004). The prevalence of these strains has increased from 6.5% in 2000-2002 (Nyangao*et al.,* 2010) to 7.1% in this study period. Sequencing of these specimens would provide additional information on the genetic diversity of serotype G2 strains in Africa.

In this study, the usual genotypes made up 28.3% of cases, whereas unusual genotypes, including possible reassortants and animal strains, were responsible for 19.0% of infections, and 30.6% of specimens could not be genotyped using the primer set. In comparison, Nyangao and colleagues (Nyangao*et al.,* 2010) detectedusual genotypes in 30.5% of cases, unusual genotypes, including possible reassortants and animal strains in 15.7% of infections, and specimens revealing mixed infections in 9.2% of cases in the 2000-2002 study in Kenya. Interestingly, Iturriza-Go´mara and colleagues (Iturriza-Go´mara*et al.,* 2001) detected reassortants in only 2% of isolates in a study conducted in the United Kingdom between 1995 and 1999. Such reassortmentis thought to be generated by frequent reassortment of the genome segments and interspecies transmission of rotaviruses between humans and animals (Nakagomi and Nakagomi, 2002).Since animal-human reassortment seems to occur in areas with significant animal–human interaction, a situation common in developing countries such as Kenya, it would be necessary to sequence the unusual genotypes isolated in this study to help determine anydegree of natural reassortment in Kenyan communities. (Cunliffe *et al.*, 2009).

The failure to identify genotype in 30 and 42 specimens with the G-specific and P-specific primers, respectively, may have been due to too little RNA or its destruction during extraction by phenol/chloroform (Pennap*et al.,* 2000) or the failure of the genotyping primer set used. Although phenol/chloroform is an inexpensive and quick method for extracting dsRNA from stoolspecimens, PCR reactions may often fail owing to the presence of PCR inhibitors that have not been adequately removed using this reagent. The alternative column-based extraction methodsare more expensive and requiremore time to complete but do tend to remove more PCR inhibitors and enable better genotyping results (data not shown).

5.6 Conclusions

The detection of rotavirus infections at a significantly high rate of 36.6% of all diarrhoeal cases among children attending selected health facilities in Kiambu District ofKenyareveals the importance of rotavirus infections in the overall burden of diarrhoeal diseases in children below the age of five years.

Rotavirus infections occurred among children of all age groups between 1 month and 5 yearsattending selected health facilities in Kiambu District ofKenya,with a peak at 6 to 8 months of age. There was a low rate of rotavirus isolation among the infants aged below 6 months and a drastic reduction in the number of cases among the children above 2 years of age.

The fact that rotavirus infections persisted year-roundwith slight peaks and valleys in some months as observed in this and other related studiesin tropical areas of the world, and that rotavirus responds to climatic changes in many different climatic zones throughout the world, may suggest that it is not an absolute temperature or rainfall level that rotavirus prefers, but rather a relative change in climatic conditions. Such environmental factors probably play a role in organizing the peak of rotavirus transmission to times when conditions are most favourable to virus survivability and transmission. Thus, paying close attention to local climatic conditions may help improve our understanding of the transmission and epidemiology of rotavirus disease.

Since the overall electropherotypic pattern produced is a reproducible trait of a rotavirus strain, the heterogeneity of electropherotypes observed in this study provides vital information on genetic diversity of rotavirus strains circulating in Kiambu district.Such information may

beuseful epidemiological indicators on the evolution,transmissions and spread of rotavirus strainsthrough the study population.

The detection of more than 11 RNA segments in some specimen may suggest the possibility of simultaneous infection by more than one rotavirus electropherotype and/or the occurrence of modification in the length of RNA segments during infection. The implication of this phenomenon is that reassortant strains are likely to emerge following the simultaneous infection of an individual with different isolates, which may play a major role in generating strain diversity within the study region.

This study has confirmed the continued importance of globally (G1-G4 and G9) and regionally (G8 and P6) predominant rotavirus serotypes in causing diarrhea among children in Kiambu District ofKenya. The study has also identified at least 10 different P-G combinations and emergence of unusual rotavirus strains (G2P[8], G4P[4], G9P[4] and G8P[4]). The finding of this enormous diversity among rotavirus strains provides insight into the evolution of rotavirus strains and creates a new challenge for rotavirus vaccine programs.

The GAVI Alliance iscurrently accepting applications for the funding of rotavirus vaccines in GAVI-eligible countries in Eastern Europe and South America (PATH, 2007). However, additional vaccine efficacy data is required before funding of these vaccines for developing countries in Africa and Asia will be considered. To this end, information on burden of disease and circulating strains in these regions will be required. Therefore, the detectionand characterization of rotavirus strains circulating in Kiambu district in Kenya between 2008 and

2011 adds to the baseline data on the molecular epidemiology of rotaviruses in the country. Such information is expected to aid in seeking advocacy for vaccine introduction in the country's national immunization program and to support an informed and evidence-based decision about the suitability of a particular vaccine with regard to the circulating genotypes and for future evaluation of the efficacy of the introduced vaccine.

5.7 Recommendations

Following the high prevalence rate of rotavirus infections observed in this and other related studies in Kenya, there is urgent need to establish high-impact and cost-effective public health intervention tools, key among them being theintroduction of rotavirus vaccine into the national immunization programme, to greatly reduce the number of deaths due to diarrheal diseases, greatly reduce the burden on the health system and to help achieve Millennium Development Goal 4.

The heterogeneity and ever-changing epidemiology of rotavirus observed in this and other related studies underscores the need for continued surveillance of rotavirus strains throughout Kenya through large-scale studies to ensure that vaccination programs provide optimal protection.

Further studies of longer duration are preferable for establishment of the relationship between climate and rotavirus disease, which may increase our understanding of the transmission and epidemiology of rotavirus disease.

It is also recommended that an additional set of genotyping primers or sequencing be used to genotype the existing rotavirus-positive specimens that were non-typeableby the primer set used in this study.

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